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The role of the A1 adenosine receptor in protecting the myocardium from ischaemia/reperfusion injury

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The Role of the A_1 Adenosine Receptor in Protecting the Myocardium From Ischaemia/Reperfusion Injury

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The Role of the A_1 Adenosine Receptor in Protecting the Myocardium From Ischaemia/Reperfusion Injury

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Jasmin Bhandal

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This is to certify that the above named applicant has completed the Coventry University Ethical Approval process and their project has been confirmed and approved as Medium Risk

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Abstract

Activation of A_1 adenosine receptors have previously been researched to protect the myocardium from ischaemia reperfusion injury in various animal models throughout the pre-conditioning phenomenon. The PI3K-AKT and MEK1/2-ERK1/2 cell survival pathways have been shown to play a critical role in the regulation in myocardial ischaemia reperfusion injury. In this study we investigated whether the A_1 adenosine receptor agonist 2'-MeCCPA protects the myocardium from ischaemia reperfusion injury when administered at the onset of reperfusion or post-reperfusion and whether the protection involved the PI3K-AKT or MEK1/2-ERK1/2 cell survival pathways and if this protection was definitely mediated through the A_1 adenosine receptor. In the Langendorff model of ischaemia reperfusion injury, the isolated perfused rat hearts underwent 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. The administration of 2'-MeCCPA (10nM) at the onset of reperfusion significantly decreased infarct size to risk ratio in comparison to non-treated ischaemic reperfused control hearts. This protection was abolished in the presence of the PI3K inhibitor Wortmannin or MEK1/2 inhibitor UO126; as well as in the presence of A_1 adenosine antagonist DPCPX. Western blot analysis determined that the administration of 2'-MeCCPA (10nM) upregulated AKT phosphorylation. In the adult rat cardiomyocyte model of hypoxia/reoxygenation, cells underwent 1 hour of hypoxia and 3 hours of reoxygenation. Administration of 2'-MeCCPA (10nM) at the onset of reoxygenation significantly decreased cellular apoptosis and necrosis. The administration of 2'-MeCCPA (10nM) in the presence of Wortmannin, UO126 and DPCPX significantly reversed the anti-apoptotic and anti-necrotic effects.

The methods used within this study include the Langendorff technique to investigate cardioprotection within the ischaemia/reperfusion model of an isolated rat heart. Apoptosis and necrosis levels were investigated as well as cleaved caspase-3 activity within the hypoxia/reoxygenation model of isolated rat cardiomyocytes. Western blots were also used to investigate the AKT phosphorylation levels within this study.

Our data furthermore implied that 2'-MeCCPA protects myocytes that were subjected to hypoxia/reoxygenation injury via decreasing cleaved caspase-3 activity that was also

abolished in the presence of PI3K inhibitor Wortmannin as well as in the presence of the MEK1/2 inhibitor UO126 and A_1 adenosine antagonist DPCPX.

Interestingly, postponing the administration of 2'-MeCCPA to 15 or 30 minutes after the onset of reperfusion significantly protected the isolated perfused rat heart from ischaemia reperfusion injury in a Wortmannin, UO126 and DPCPX sensitive manner. This protection was associated with an increase in AKT phosphorylation.

Administration of the A_1 adenosine receptor agonist 2'-MeCCPA 15 or 30 minutes after the onset of reoxygenation significantly protected isolated adult rat cardiomyocytes that were subject 1 hour of hypoxia and 3 hours of reoxygenation from injury in an anti-apoptotic/necrotic manner. This anti-apoptotic and necrotic effects was abolished upon the administration of PI3K inhibitor Wortmannin, MEK1/2 inhibitor UO126 and A_1 adenosine antagonist DPCPX. Delaying the administrations of 2'-MeCCPA to 15 or 30 minutes after reoxygenation was also associated with a decrease in cleaved caspase-3 activity which was abolished in the presence of PI3K inhibitor Wortmannin; however MEK1/2 inhibitor UO126 was only able to abolish protective effects at 15 minutes and no effect at 30 minutes post-reoxygenation.

It can be implied for the first time that the administration of 2'-MeCCPA at the onset of reperfusion protects the ischaemic reperfused rat myocardium from lethal ischaemia reperfusion injury in a PI3K and MEK1/2 sensitive manner. Delaying the administration of 2'-MeCCPA to 15 minutes or 30 minutes after the onset of reperfusion or reoxygenation, it significantly protects the isolated perfused rat heart from ischaemia reperfusion injury and also the adult rat cardiomyocyte from hypoxia-reoxygenation injury in an anti-apoptotic/necrotic manner. Moreover, this protection is associate with recruitment of the PI3K-AKT and MEK1/2-ERK1/2 cell survival pathways.

Furthermore, any protective effects observed with the administrations of 2'-MeCCPA at the onset of reperfusion, 15 minutes or 30 minutes post-reperfusion/reoxygenation was significantly abrogated in the presence of the A_1 adenosine antagonist DPCPX which implies that protection was definitely occurring through the A_1 adenosine receptor subtype.

The work carried out in this thesis has implied the cardioprotective effects of the activation of A_1 adenosine receptors in an ischaemia/reperfusion injury model. This is the first study to

imply the delayed activation of A_1 adenosine receptors with A_1AR agonist 2'-MeCCPA to confer cardioprotection in isolated perfused rat hearts. The findings from this study imply clinically important developments in the field of managing myocardial infarction. Urgent studies are required to investigate the potential role of A_1 adenosine receptor agonists in ameliorating myocardial ischaemia reperfusion injury in human.

Publications and Presentations from this PhD Thesis

Bhandal, J., Buckley, J., Renshaw, D., Maddock, H., and Hussain, A. (2016) Post-Reperfusion Activation of A_1 Adenosine Receptors Protect the Myocardium from Ischaemia Reperfusion Injury. PA2 Online - E-Journal of the British Pharmacological Society P34 **Abstract, Poster and Oral Presentation at British Pharmacological Society Annual Meeting, December 2016. London, United Kingdom**

Bhandal, J., Buckley, J., Renshaw, D., Maddock, H., and Hussain, A. (2017) A_1 Adenosine Receptor Activation Can Protect the Myocardium from Ischaemia Reperfusion Injury throughout Post-Reperfusion **Abstract and Poster Presentation at International Society of Heart Research, June 2017, Hamburg, Germany**

Bhandal, J., Renshaw, D., Buckley, J., Maddock, H. and Hussain, A. (2017) P1747A1 Adenosine Receptor Activation Can Protect the Myocardium From Ischaemia Reperfusion Injury Post Reperfusion. *European Heart Journal* 38 **Abstract and Poster Presentation at European Society of Cardiology, August 2017. Barcelona, Spain**

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List of Abbreviations

2'-MeCCPA	A1 adenosine receptor agonist
8-SPT	Unselective adenosine receptor antagonist
A_1AR	A1 adenosine receptor subtype
A_{2A}AR	A2A adenosine receptor subtype
A_{2B}AR	A2B adenosine receptor subtype
A_3AR	A3 adenosine receptor subtype
AAR	Area at risk
ACE	Angiotensin Converting Enzyme
ADP	Adenosine Diphosphate
AIF	Apoptosis Inducing Factor
AKT	Cellular AKT
AMP	Adenosine Monophosphate
APAF1	Apoptosis Protease Activating Factor 1
ARC	Apoptosis Repressor caspase Recruitment Domain
ATP	Adenosine Triphosphate
BAD	BCL 2 family pro-apoptotic protein
BAK	BCL 2 family pro-apoptotic protein
BAX	BCL 2 family pro-apoptotic protein
BCL2	B-cell leukaemia/lymphoma 2
BCLxl	Bcl-2-like 1 protein
BID	BCL 2 family pro-apoptotic protein
BIM	BCL 2 family pro-apoptotic protein
BSA	Bovine Serum Albumin+C17
CABG	Coronary Artery Bypass Grafting
CASPASE	Cysteine Aspartate Albumin
CPA	A1 Adenosine Receptor Agonist
CCPA	A1 Adenosine Receptor Agonist
CHD	Coronary Heart Disease
CF	Coronary Flow
CL-IB-MECA	A3 Agonist
CO_2	Carbon Dioxide
CVD	Cardiovascular Disease
DAG	Diacetylglycerol
DNA	Deoxyribose nucleic Acid
DPCPX	A1 adenosine receptor antagonist
ERK	Extracellular Regulated Kinase
ER	Endoplasmic Reticulum
eNOS	Endothelial Nitric Oxide Synthase
FACS	Fluorescent Assisted Cell Sorting
FADD	Fas Associated Death Domain
FASL	FAS Ligand
FLIP	FADD Like Inhibitory Proteins

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-Protein	G-protein regulatory protein
H_2O_2	Hydrogen Peroxide
Hyp/Reox	Hypoxia/Reoxygenation
IAP	Inhibitor of Apoptosis protein
IAP 1	Inhibitor of Apoptosis 1
IAP 2	Inhibitor of Apoptosis 2
IB-MECA	A3 Agonist
IR	Ischaemia/Reperfusion
JNK/SAPK	C-Jun C-terminal kinase/stress-activated protein kinase
KHB	Krebs Henseleit Buffer
KO	Knockout
LVDP	Left Ventricular Developed Pressure
MAPK	Mitogen activated protein kinase
MEK1/2	Mitogen Extracellular Kinase 1/2
MLKL	mixed lineage kinase domain-like
MPTP	Mitochondrial permeability transition pore
MI	Myocardial Infarction
mPTP	Mitochondrial permeability transition pore
NECA	Non-selective adenosine agonist
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2	Oxygen
PDK1	Phosphoinositide-dependant kinase 1
PIA	A1 adenosine receptor agonist
PKA	Protein Kinase A
PKC	Protein Kinase C
PI3K	Phosphatidylinositol 3 kinase
PLC	Phospholipase C
RAF	MAP kinase kinase kinase
RISK	Reperfusion injury Salvage kinase pathway
RIP1	Receptor-interacting protein 1
RIP3	Receptor-interacting protein 3
ROS	Reactive Oxygen Species
SAFE	Survival activating factor enhancement
SEM	Standard Error of the MEAN
SER	Serine
SMAC/DIABLO	Second mitochondrial activator of caspases
SR	Sarcoplasmic Reticulum
TTC	2,2,3 triphenyltetrazolium chloride
TYR	Tyrosine
UO126	MEK1/2 Inhibitor
Wort	Wortmannin

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Chapter 1: Introduction

1.1 Cardiovascular Disease

Cardiovascular diseases (CVDs) are yet to remain the single leading causes of global deaths worldwide with a constant increase in mortality and morbidity and a projected number of deaths expecting to increase to almost 23.6 million by the year 2030 (WHO 2020). Within the UK alone, there are around 7.4 million people living with CVDs (British Heart Foundation 2020). More than a quarter of deaths in the UK are due to CVDs meaning nearly 170 000 deaths per year; this is an average of 460 deaths per day or one death every three minutes (British Heart Foundation 2020).

CVDs encompass a variety of disorders that target the cardiovascular system. These include; coronary heart disease (CHD), cerebrovascular disease, peripheral arterial disease, rheumatic heart disease and venous thromboembolism (WHO 2017, Stewart, Manmathan and Wilkinson 2017). Acute events of the heart are mainly caused by occlusions that can in turn prevent the blood flowing to the heart and the brain (WHO 2017), this has resulted in serious health concerns, physical disabilities and deaths (Nichols et al. 2013).

The main behavioural risk factors that can lead to the development of CVDs include; an unhealthy diet leading to obesity, tobacco use and an unhealthy use of alcohol (WHO 2017). These behavioural risk factors can express; a raised blood pressure causing hypertension, a raised blood glucose level resulting in diabetes mellitus and increased levels of low density lipoproteins (LDLs) and a low level of high density lipoproteins (HDLs) (WHO 2017, Lucero et al. 2014).

Significant primary interventions have evolved in order to manage cardiovascular risks within the population and although these are reliable in being able to prevent coronary artery occlusion and in turn prevent acute events of a heart attack, it is also imperative that more robust and enhanced secondary interventions are put in place, especially after an ischaemic insult (WHO 2007).

1.2 Myocardial Infarction

Myocardial infarction (MI), which can otherwise be termed as a heart attack, can be defined as the sudden ischaemic death of myocardial tissue (Neri et al. 2017). A myocardial infarction is when atherosclerotic plaque formations occur within the walls of arteries and in turn can cause reduced blood flow to the myocardium and therefore cause a lack of oxygen and nutrients to the heart (Lu et al. 2015). Atherosclerosis is a condition that can also form due to a build-up of fatty deposits in the arterial walls and further causing myocardial infarction (Reynold 2012). On commencement of a MI the symptoms consist of; chest pain, arrhythmia and shortness of breath, which can lead to permanent damage of the heart muscle (Lu et al. 2015).

Research has led to the better understanding of the pathogenesis of acute coronary disorders such as angina and MI (Grundy 1999). Effective treatments that allow for the management of these conditions include surgical interventions and also the administration of drug therapies such as aspirin (an anti-platelet drug), warfarin (an anticoagulant drug), statins (drugs that lower endogenous cholesterol synthesis), beta blockers as well as angiotensin converting enzyme (ACE) inhibitors (Anderson et al. 2007). These treatments are all secondary treatments in order to manage CHD (Anderson et al. 2007). Ultimately, for those patients who have withstood a MI will require treatments during the reperfusion period where medical treatments restore the blood flow to any occluded arteries to further restore somewhat normal functioning (Kloner and Rezkalla 2004).

1.3 Myocardial Ischaemia

Myocardial ischaemia can be caused by a deficit in blood supply to the myocardium due to the obstruction within arterial flow which can lead to occlusion of the vessels that supply the myocardium (Hausenloy and Yellon 2013; Neri et al. 2017). This causes an increase in oxygen demand and a decrease in actual oxygen supply due to the lack of blood flow. This is an important factor that contributes to heart failure (Hausenloy and Yellon 2013).

Prolonged ischaemia can result in a metabolic changes due to a lack of oxygen and nutrients supply however it can also lead to ultrastructure changes within the cells of myocardium tissue. These changes include; alterations in membrane potential and ion distribution, cellular

swelling and rupturing, decreased ATP supply to cells, changes in free radical mediated injury, disruptions in oxidative phosphorylation, elevated levels of Ca^{2+} within the mitochondria and increased levels of lactic acid causing a decline in intracellular pH and altered metabolism (refer to Figure 1.0) (Hausenloy and Yellon 2013; Halestrap et al. 2004; Allen et al. 2008). These serious alterations can further lead to cell death via the mechanisms of necrosis, apoptosis and autophagy (Kalogeris et al. 2012).

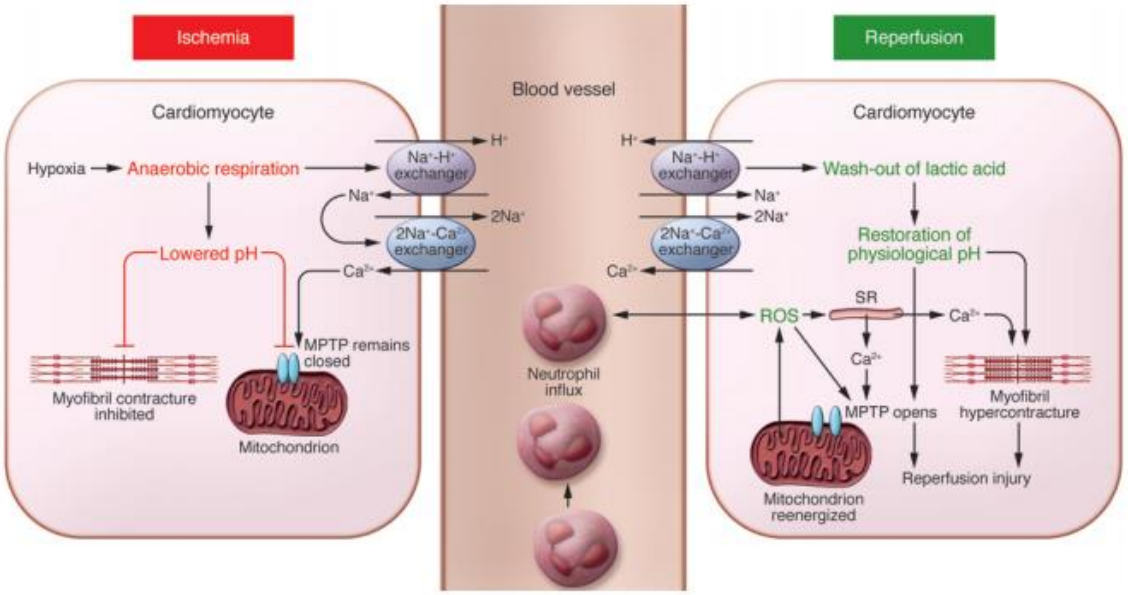


Figure 1.1 Schematic diagram that illustrates the components of myocardial ischaemia-reperfusion injury (IRI). The lack of oxygen present within the cell causes a switch to anaerobic respiration which results in the build-up of lactic acid which in turn causes a drop in cellular pH. This causes the Na^+-H^+ exchanger, between the cell and the blood supply, extrude H^+ out of the cell and cause an overload of intracellular Na^+. This process activates the $2Na^+$-Ca^{2+} exchanger which then extrudes the Na^+ out of the cell and causes a further overload in intracellular Ca^{2+}. The acidic conditions prevent the MPTP from opening and further prevent hyper contracture of the cardiomyocyte cell from occurring. During reperfusion, the Na^+-H^- exchanger is reactivated which causes the lactic acid to wash-out and in turn allow for the restoration of the cellular pH. The electron transport chain is also reactivated which allows for the development of ROS which can subsequently trigger the MPTP opening and allow for myofibril hyper contracture to occur which utilises the Ca^{2+} overload that occurred when ischaemia was taking place (Hausenloy and Yellon 2013).

1.4 Myocardial Reperfusion

Myocardial reperfusion is a vital phenomenon in which cardiomyocyte recovery and maintenance is facilitated due to the neutralisation of the lactic acid build up and the reactivation of the Na^+-H^+ exchanger which then goes on to restore cellular physiological pH levels (Hausenloy and Yellon 2013) (refer to Figure 1.1). This occurs due to the reintroduction of coronary blood flow to the myocardium after the ischaemic event, however this restoration of blood flow can additionally result in reperfusion-induced injury (Kalogeris et al. 2012).

Reperfusion can therefore been seen as beneficial for the myocardium after an ischaemic event as it rescues the myocardium from the deleterious effects as described in Figure 1.1; however alongside this, reperfusion of an ischaemic myocardium can also come with detrimental consequences which further results in cardiomyocyte injury and death which is called 'ischaemia-reperfusion injury' (Derek and Hausenloy 2007).

Previous studies have described reperfusion as a period in which medical interventions can be applied in order to reduce injury to myocardium tissue (Yellon and Hausenloy 2013). Treatments within reperfusion have resulted in much success and also an effective way of managing ischaemia and in the long run very beneficial (Kloner and Rezkalla 2004). Although research has provided the information that early reperfusion is the best time period in which ischaemic injury can be reduced, the restoration of the blood flow to the ischaemic myocardium can consequently cause a burst of reactive oxygen species (ROS) as well as inflammatory mediators. This can occur naturally in biological systems and play a major role in ischaemia-reperfusion injury (Hausenloy and Yellon 2013; Zhou et al. 2018).

The event of early myocardial reperfusion with the aid of thrombolytic therapies or primary percutaneous coronary interventions are proven to be most successful strategies in managing myocardial infarction and in turn improving clinical outcomes (Yellon and Hausenloy 2007).

Despite the major benefits that comes with reperfusion, the resulting reperfusion injury comes with its many negative attributes too. These include; arrhythmias, myocardial stunning which can cause further reversible and/or irreversible cellular damage, as well as the release of free radicals within the ischaemia and reperfusion periods (Kalogeris et al. 2012).

Previous studies have also mentioned that the progression of a myocardial injury can be enhanced by the immune system which include cell types such as neutrophils, macrophages and mast cells (Fitridge and Thompson 2012). A previous review written by Epelman and Mann (2012) stated that neutrophils are the most abundant leukocyte within the systemic circulation thus adding to the myocardial injury causing neutrophil-mediated cardiac injury. When a myocardial infarction occurs, the endothelium activates through a variety of cytokines and this causes an upregulation of neutrophils to the injury site. These neutrophils then bind to ICAM-1 which is a protein expressed by cardiomyocytes thus causing an intense trigger of oxidative stress and therefore enhanced myocardial injury (Epelman and Mann 2012). Neutrophil-mediated myocardial injury increases as the ischaemic period increases (Epelman and Mann 2012; Fitridge and Thompson 2012).

1.5 Oxygen-Derived Free Radicals

Oxygen derived free radicals can be introduced once the coronary blood flow to the myocardium is restored after the ischaemic event. This restoration of blood flow leads to a further restoration of oxygen to the myocardium thus including the generation of oxygen metabolites in the form of ROS (Braunersreuther and Jaquet 2012).

ROS take the form of chemically reactive ions, radicals and molecules that form from oxygen. The majority of free radicals are formed within the mitochondria during the reperfusion period (Camara 2009). ROS are small molecules that are generated by oxygen reduction which can lead to a presence of unpaired electrons upon the oxygen molecule that has been reduced. This addition of an electron to the oxygen molecule (O_2) can form a superoxide anion radical ($O_2^{\bullet -}$); this is considered to be the primary form of ROS (Braunersreuther and Jaquet 2012). These superoxide radicals have a very short half-life and can interact directly or through enzyme- or metal-catalysed reactions with other molecules to generate a secondary ROS (Braunersreuther and Jaquet 2012).

Throughout normal physiological conditions of the myocardium, an enzyme named xanthine oxidoreductase is present in a dehydrogenase form and simply acts as a rate-limiting step within the purine degradation to uric acid (Jankov et al. 2008). Consequently, once ischaemic conditions commence, ATP is broken down into purine hypoxanthine which is a substrate for an enzyme named xanthine oxireductase; this further cause's reversible oxidation or

irreversible proteolytic cleavage which causes the conversion of xanthine oxireductase to xanthine oxidase. This is primarily causes the deleterious formation of ROS as well as hydrogen peroxide (Pritsos 2000; Braunersreuther and Jaquet 2012).

Throughout ischaemic periods, oxygen-derived free radicals are formed to such destructive levels during the reperfusion period of the previously ischaemic region which can cause inhibition of contractile function within myocardium tissue thus enhancing myocardial injury (Zweier and Talukder 2006). Although ROS possesses such consequential attributes within the myocardium and is well documented in favour of cellular damage, it also does participate in a range of other roles such as within cell signalling as well as microbial killing (Stowe and Camara 2009; Rada and Leto 2008).

A study carried out by Hoshikawa and colleagues (2001) researched that when male Sprague Dawley rat hearts were exposed to hypoxic conditions, there was a constant increased level of xanthine oxidase hypoxanthine which is a pathway known to generate oxidative stress *in vivo* (Hoshikawa et al. 2001).

Although ischaemia reperfusion injury involves the production of ROS, an accumulation can result is cellular oxidative stress, dysfunction of the mitochondria, initiation of cell death and the activation of the mitochondrial permeability transition pore. In order reduce the effects of ROS, antioxidant therapies have been shown to provide positive treatment outcomes to reduce ischaemia-reperfusion injury (Zhou et al. 2018). With ROS acting as an important signalling molecule to involve the immune system to respond to the injury signals, endogenous antioxidants such as catalase, superoxide dismutase (SOD), glutathione and glutathione peroxidase (GPx) are important scavengers that are able to maintain ROS at normal levels (Zhou et al. 2018). During specific pathological conditions such as an ischaemic event, antioxidant defences can be overwhelmed and in turn result in oxidative stress and an uncontrollable amount of ROS production (Zuo et al. 2015; Zhou et al. 2018). Investigations have been conducted upon antioxidants in alleviating ischaemia-reperfusion injury. A study by Adlam and colleagues (2005) investigated mitoQ, a selective mitochondrial ROS inhibitor that has the ability to dramatically attenuate heart dysfunction after ischaemia-reperfusion in rats (Adlam et al. 2005).

1.6 Myocardial Stunning

Myocardial stunning is another negative consequence that can enhance ischaemia-reperfusion injury (Hausenloy and Yellon 2013). This is when post-ischaemic contractile dysfunction is introduced to the myocardium at the onset of reperfusion. This phenomenon can be detrimental in terms of initiating oxidative stress and calcium overload (Hausenloy and Yellon 2013; Kloner et al 1998).

1.7 No-Reflow Phenomenon

The no-reflow phenomenon essentially occurs at the point of reperfusion when the occluded coronary artery becomes free of stress and in an ideal situation, the blood flow to the ischaemic tissue is supposed to flow through immediately; however due to detrimental changes that have occurred in the cardiac capillaries and arterioles, the blood flow becomes impeded (Reffelmann 2002; Hausenloy and Yellon 2013). Sufficient structural damage has occurred to the microvasculature and therefore this causes a cease in restoration of normal blood flow to the cardiomyocytes. There is inadequate healing of cardiac tissue and therefore this can affect future blood flow (Hausenloy and Yellon 2013).

1.8 Metabolic Changes

Research has currently established that there are multifactorial mechanisms that contribute to ischaemia-reperfusion injury when a myocardial infarction occurs (Kalogeris et al. 2012). The metabolic changes that occur are important; these include the accumulation of lactic acid that results from an increased rate of glycolysis which further decreases the intracellular pH within the cardiomyocyte (Halestrap et al. 2007). This occurs due to conditions changing from aerobic respiration to anaerobic respiration (Halestrap et al. 2007). A rapid decrease in available adenosine triphosphate (ATP) levels occurs which leads to the inhibition of the Na^+/K^+ ATPase, which can then cause a rapid increase in intracellular Na^+ which further link to increases in Ca^{2+} in the cell (Kalogeris et al. 2012; Hausenloy and Yellon 2013; Halestrap et al. 2004)

1.9 Calcium Overload

An increase in cytosolic Ca^{2+} can contribute to the exacerbated effects of ischaemia-reperfusion injury. Throughout the ischaemic period, the intracellular levels of Ca^{2+} rise due to the Na^{+}/H^{+} exchanger extruding H^{+} ions in exchange for Na^{+} ions (Hausenloy and Yellon 2013). The increase in Na^{+} ions are then exchanged for Ca^{2+} via the Na^{+}/Ca^{2+} exchanger within the plasma membrane of cardiomyocytes (which usually pumps Ca^{2+} out of the cell) (Hausenloy and Yellon 2013; Kalogeris et al. 2012; Bains 2009). These deleterious effects are contracture and death (Kalogeris et al. 2012). The sarcoplasmic reticulum (SR), which is a Ca^{2+} store, is also affected during ischaemia-reperfusion injury and does not allow for the reuptake of Ca^{2+} (Kalogeris et al. 2012).

Cardiac contractility is regulated by changes in Ca^{2+}concentration and for normal functioning, Ca^{2+} concentrations are sufficiently high in systole and low in diastole. Most of the Ca^{2+} that is needed for contraction comes from the SR (Eisner et al. 2017). The SR functions as a dynamic calcium governor in which an automatic feedback control for altering and maintaining myoplasmic and SR calcium levels is achieved (Rossi and Dirksen 2005). The way in which this automatic feedback is achieved is by the interactions between the terminal regions of the SR and the transverse tubular membrane alongside the envelopment of the myofibrils from the longitudinal SR (Rossi and Dirksen 2005). The SR plays a central role in controlling Ca^{2+} cycling and with this the SR has developed a detailed set of calcium-regulatory proteins that are able to provide a release of Ca^{2+} upon excitation (systole) and also provide a means of Ca^{2+} reuptake with a maintained calcium storage when muscle is relaxed (diastole) (Rossi and Dirksen 2005). This can be referred to as excitation-contraction (EC) coupling (Gorski et al. 2015).

During systole, an action potential causes depolarisation of the sarcolemma which can result in the entry of a small amount of extracellular calcium into the cytosol through voltage gated L-type calcium channels. The calcium then binds to ryanodine receptors that triggers a larger efflux of calcium from the SR and into the cytosol (Gorski et al. 2015). This tenfold increase of intracellular calcium activates calcium sensitive contractile proteins like troponin C, which further uses ATP to produce tension and muscle contraction. Furthermore, for muscle relaxation to occur, calcium is removed from the cytosol by the sodium-calcium exchanger as

well as ATPase and pumped back into the SR (Gorski et al. 2015). For heart failure to commence there are pronounce cellular changes that occur and this is due to an increase in end-diastolic cytosolic calcium levels. This means SR calcium reuptake decreases due to the SERCA2a modulate that allows for calcium reuptake becomes dysfunctional (Gorski et al. 2015).

It's the negative alterations of Ca^{2+} are what contribute towards cell death during ischaemia-reperfusion injury and one of the ways in which cardiomyocytes can try and deal with the lethal increase in Ca^{2+} is by the mitochondrial Ca^{2+} uniporter taking up the increased levels of Ca^{2+}; this is called the mitochondrial permeability transition (MPT) response (Kalogeris et al. 2012). This increase in Ca^{2+} can also cause the activation of the calmodulin-dependent protein kinases (CaMKs) which also contribute towards ischaemia-reperfusion injury (Kalogeris et al. 2012).

The Ca^{2+} overload is often accompanied by hyper activation and consistent hyper contracture within cardiomyocytes; and with this consecutively occurring cell rupture can occur and in turn majorly increasing Ca^{2+} overload as seen in Figure 1.2 (Piper 2000).

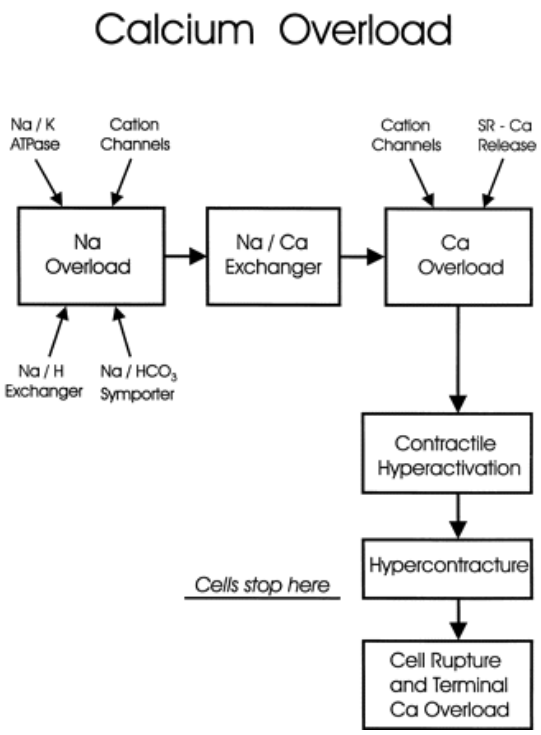


Figure 1.2 Schematic diagram of the mechanism of calcium overload that can enhance ischaemia-reperfusion injury (Piper 2000).

1.10 Cell Death

Cells have the ability to respond to stress in various ways however it is ultimately the phenomenon of cell death that eliminates the cells that are damaged due to age or through means of injury (Fulda et al. 2010). Cells death is divided up into categories consisting of; necrosis, apoptosis and autophagy (Fulda et al. 2010) as referred to in Figure 1.3. Studies have gone on to investigate the effects of ischaemia-reperfusion injury upon necrosis and apoptosis and the major pathways of cell death (Kim and Kang 2010; Eefting et al. 2004).

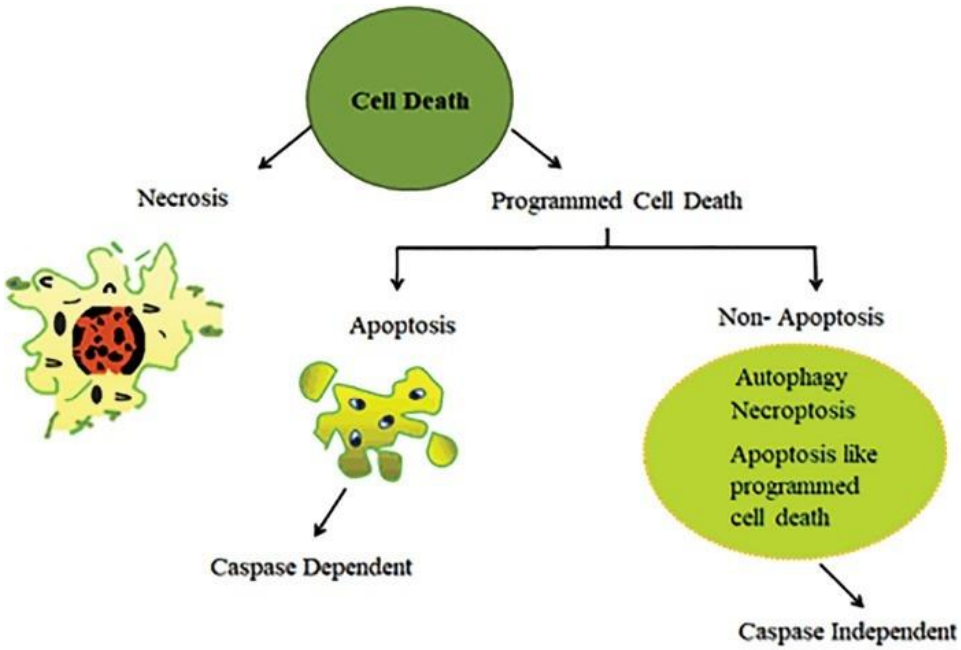


Figure 1.3 General modes of cell death which include programmed cell death and necrosis (Jan and Chaudhry 2019).

1.11 Necrosis

Necrosis is known as irreversible injury to cells and can be triggered as a response to physical and chemical insults, of which include hypoxia, ischaemia and steep changes in temperature (Galluzzi et al. 2014; Konstantindis, Whelan and Kitsis 2012). Necrosis was defined as unplanned/accidental cell death however recent studies have developed that necrosis is an active form of cell death (Bergh et al. 2014).

The process of necrosis has been defined as an oxidation-mediated necrotic pathway, it is ATP independent and involves the interactions of multiple regulatory factors. Another pathway of cell death includes an ATP dependent pathway called apoptosis (Sancho et al. 2006). A study conducted by Lieberthal et al. in 2013 showed that when cultured mouse proximal tubular cells showed an increase in cell death, this was negatively proportional to the amount of ATP released showing that an increase in cell death was associated with a decrease in ATP availability; meaning that necrosis was independent of ATP release (Lieberthal et al. 2013).

For many years it was proposed that necrosis is the only process of cell death that is implicated within cardiomyocytes following ischaemia-reperfusion injury; however since then significant research was conducted and other forms of cell death were elucidated. These areas being, apoptosis, autophagy and oncosis (Buja and Vela 2008; Kostin et al. 2003). The main cause of necrosis occurs when the myocardium is undergoing reperfusion and the opening of the MPTP causes dysfunction of the mitochondria and therefore enhances cardiomyocyte necrosis (Hausenloy 2012; Hausenloy and Yellon 2013).

1.12 Apoptosis

Apoptosis in an ATP-dependent, highly conserved biochemical process that becomes activated under physiological and pathological circumstances in response to a stimuli (Fulda et al. 2010). Apoptosis is also known as programmed cell death (Elmore 2007; Fulda et al. 2010). When apoptosis occurs, cell morphology changes in such ways that cell shrinkage occurs, cytoplasmic condensation and cellular fragmentation into membrane-enclosed apoptotic bodies also occur (Fulda et al. 2010; Konstantinidis, Whelan and Kitis 2012). These apoptotic bodies are then rapidly phagocytised by macrophages and eliminated (Elmore 2007; Konstantinidis, Whelan and Kitis 2012).

Much research has been carried out to identify and elucidate the molecular mechanisms that regulate and further execute the process of apoptosis (Kim and Kang 2010). Studies have determined that apoptosis is a tightly regulated process and there are various interactions between a ranges of pro- and anti-apoptotic molecules. A number of different proposed mechanisms have been identified and characterised to define apoptosis. These include the intrinsic vs. extrinsic pathway or the caspase-dependent vs. caspase-independent pathway (Kim and Kang 2010).

Caspases originate from a family of cysteine proteases and are produced as zymogens that when become activated, their pro-domains are cleaved off (Nicholson and Thornberry 1997; Kim and Kang 2010). There are different categories of caspases and they are grouped together based upon their structure which determines their function. Initiator caspases hold long pro-domains and examples of these are caspase-8 and -9; which are able to initiate apoptosis and also downstream themselves to activate effector caspases (Kim and Kang 2010). Effector caspases are characterised with short pro-domains, an example of an effector caspase would be caspase-3. Caspase-3 would generally depend on an initiator caspase in order to become activated (Kim and Kang 2010). The collective results of a number of independent studies suggest that caspases can be tissue dependent and specific caspases, when activated can mediate apoptosis (Kim and Kang 2010). Caspase-mediated apoptosis recruits either the intrinsic or the extrinsic pathway however both include the common effector caspase-3 (Kim and Kang 2010).

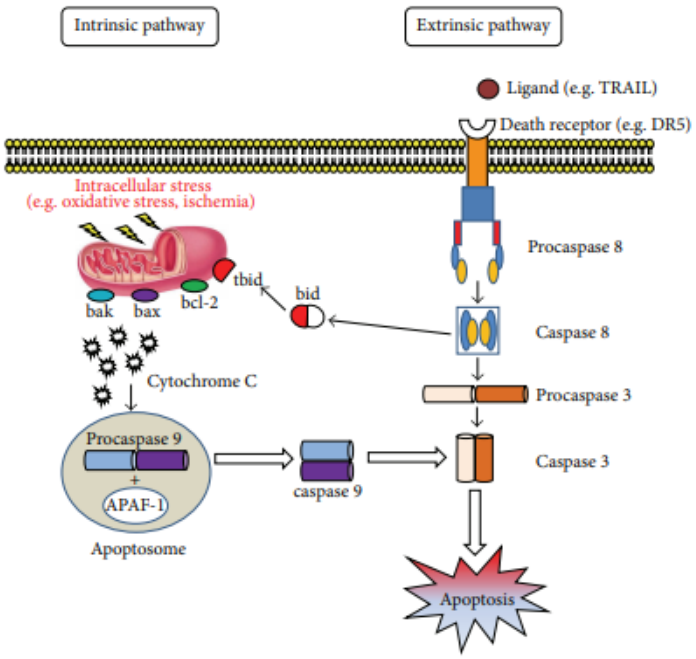


Figure 1.4 Apoptosis activates through two main pathways, the intrinsic pathway and the extrinsic pathway (Loreto 2014) the extrinsic pathway activates via the binding of death ligands (TRAIL) to death receptors. The formation of death inducing signalling complex (DISC) forms which binds to procaspase-8, this stimulates the activation of caspase-8 and further triggers the protease cascade which leads to apoptosis. The intrinsic pathway is activated via stress stimuli for example DNA damage, ER stress and hypoxia. The death signal is detected by Bcl-2 family proteins (BAD and BAX). This then leads to a conformational change in BAX and BAK causing the formation of mitochondrial pores and allowing permeability to the outer mitochondrial membrane. This promotes the release of

apoptogenic proteins (cytochrome c and SMAC/DIABLO) which interact with Apaf-1 to enable the formation of apoptosome complex which then triggers procaspase-9. Activated caspase-9 causes a cascade to activate caspase-3, -6 and -7 with the overall objective to cause apoptosis (Loreto 2014).

1.12.1 Extrinsic Apoptotic Pathway

The extrinsic pathway of apoptosis can also be termed as the death receptor-mediated pathway which initiates via the binding of a death ligand, such as TNF-α, to a cell surface death receptor such as Fas (Kiechle and Zhang 2002). This activation can further cause the recruitment of the death domain known as Fas-associated death domain (FADD) which further activates caspase-8; which then forms the death-inducing signalling complex (DISC) (Kim and Kang 2010; Kiechle and Zhang 2002). This complex triggers the auto-catalytic activation of caspase-8, which in turn activates the protease cascade (caspase-3,-6 and -7) to finally cause apoptosis (Figure 1.3) (Kiechle and Zhang 2002).

Research has been conducted to show that the Fas pathway is an important mediator of apoptosis during ischaemia-reperfusion injury *in vivo*. Lee and colleagues studied that when mice were lacking Fas, they exhibited a reduction in myocardium infarct size after ischaemia-reperfusion thus making it imperative that Fas be present for apoptosis to occur through the extrinsic pathway (Lee et al. 2003; Kim and Kang 2010).

1.12.2 Intrinsic Apoptotic Pathway

The intrinsic pathway is also known as the mitochondrial-mediated apoptosis pathway and can be triggered by various endogenous and exogenous stimuli such as ischaemia, oxidative stress and endoplasmic reticulum (ER) stress. The intrinsic pathway plays a major role in the removal of damaged cells and caspase activation is closely associated with the permeabilisation of the outer mitochondrial membrane by the B-cell lymphoma protein-2 (Bcl-2) family of proteins (Kim and Kang 2010). The proteins within the Bcl-2 family can be categorised into either anti-apoptotic proteins (Bcl-2 and Bcl-xL) or the pro-apoptotic members (Bad, Bax and Bak) (Youle and Strasser 2008; Kim and Kang 2010). In regular conditions, Bax is located within the cytosol however when responding to an exogenous stimuli, Bax then translocates into the outer mitochondrial membrane within the

cardiomyocytes (Kim and Kang 2010; Tait and Green 2010; Zhang et al. 2009). Moreover, the injurious stimuli causes a conformational change in Bak which migrates to the outer mitochondrial membrane also (Konstantinidis, Whelan and Kitis 2012; Zhang et al. 2009). Due to the activation of Bax and Bak at the outer mitochondrial membrane, mitochondrial pores form and this then enhances membrane permeability (Sarvothaman et al. 2015). Alongside this permeabilisation, the release of apoptogenic proteins is promoted such as: cytochrome c and SMAC/Diablo complex, Apoptosis Inducing Factor (AIF) and Endonuclease G (Endo G) (Fan et al. 2013; Borutaite et al. 2003; Loreto et al. 2014). Cytochrome c forms an activation complex with apoptotic protein activating factor-1 (Apaf-1), this then binds to a complex in the cytosol known as the apoptosome which triggers the activation of pro-caspase-9 (Kim an Kang 2010; Keoni and Brown 2015). Procaspsae-9 then initiates the activation of effector caspases-3, -6 and -7 which then leads to cardiomyocyte apoptosis (Figure 1.3) (Elmore 2007; Loreto et al. 2014).

Studies have been carried out to look into how targeting the intrinsic pathway could reduce myocardial ischaemia-reperfusion injury and it has been found that targeting the deletion of the pro-apoptotic protein Bax can actually result in a reduction in infarct size and also improve myocardial functioning after experimental myocardial infarction has occurred (Hochhauser et al. 2007; Kim and Kang 2009). Furthermore, Mocanu, Baxter and Yellon (2000) conducted a study that examined the effect of caspase-8, -9 and -3 , when being administered during the early stages of reperfusion were able to significantly protect the myocardium from lethal reperfusion injury (Mocanu, Baxter and Yellon 2000). This is important to note as several past studies have shown caspase-3 to mediate a pivotal role within the development of cellular apoptosis namely Namura et al (1998) showing that levels of activated caspase-3 were peaking between 30-60 minutes of reperfusion.

Overall, apoptosis has been researched to play a key part in myocardial infarction damage and the use of caspase inhibitors are beneficial to protect the myocardium from ischaemia-reperfusion injury however it is feasible to conduct further research to understand the further potential mechanisms that can therefore enhance the identification of future potential targets to promote cell survival. A study conducted by Mocanu and colleagues (2000) investigated caspase activation as being a major events of apoptosis and therefore examined how caspase inhibitors (Z-VAD fmk, Z-IETD fmk and Z-LEHD fml and Ac-DEVEcmk), when

administered during reperfusion after a myocardial infarction successfully protects isolated rat myocardium from lethal reperfusion injury (Mocanu et al. 2000).

The mitochondrial permeability transition pore (MPTP) which is a transmembrane protein that resides in the mitochondrial inner membrane. This pore normally remains closed however it can open when stimulated by mitochondrial matrix Ca^{2+} accumulation which may happen in a hypoxic events. With this, oxidative stress can also increase which can further lead to apoptosis (Bernardi 2013).

1.13 Other Forms of Cell Death

Although necrosis can be stated as an uncontrollable process, studies have potentially linked necrosis to a process called necroptosis, this process involves a crossover of necrosis and apoptosis (Konstantinidis, Whelan and Kitsis 2012; Zhe-Wei, Li-Sha and Yue-Chun 2018). Necroptosis has been proposed as an important player in the pathophysiology of heart disease and ischaemia-reperfusion injury caused by myocardial infarction (Zhe-Wei, Li-Sha and Yue-Chun 2018). Necroptosis, when activated ligands of death receptors bind such as receptor-interacting protein 1 (RIP1) mediating the activation of receptor-interacting protein 3 (RIP3) and mixed lineage kinase domain-like (MLKL); these are two vital downstream mediators that inhibit caspase-8 and upstream ischaemia-reperfusion injury via necroptosis (Zhe-Wei, Li-Sha and Yue-Chun 2018; Zhou and Yuan 2014; He et al. 2016).

There are other forms of cell death that can be seen *in vitro* and *in vivo*, hence why there is such a need to study different forms of cell death (Weerasinghe and Buja 2012). Apoptosis manifests by cell shrinkage followed by break down however another form of cell death, oncosis, is the total opposite. Oncosis involves cell swelling and coagulation of the cytoplasm (Weerasinghe and Buja 2012).

1.14 Cardioprotective Strategies in Myocardial Infarction

Research suggests that although the period of reperfusion can increase reperfusion injury, it simultaneously acts as the key to the management of myocardial infarction injury. By exploring strategies that target reperfusion, there is undoubtedly a chance that survival of patients can be enhanced (Mocanu et al. 2000; Zhang and Huo 2011; Covinhes et al. 2020).

1.15 Adenosine Receptors

There are currently four adenosine receptors that are activated by receptor-dependent and –independent mechanisms. These receptors belong to the superfamily of G-protein-coupled receptors (GPCR); A_1, A_{2A}, A_{2B} and A_3 receptors and have been categorised in the way that they cause stimulatory and inhibitory actions on adenylate cyclase (Figure 1.4) as well as selectivity of respective agonists and antagonists (Borea et al. 2016; Fredholm 2014).

Studies have shown that adenosine A_1 receptors are coupled to the pertussis toxin sensitive G_i and G_o proteins and these inhibit adenylate cyclase activity (and therefore inhibit cyclic AMP (cAMP) activation) to activate phospholipase C and the opening of K^+ channels as well as the inhibition of Ca^{2+} release (Fredholm 2000; Germack and Dickenson 2004; Borea et al. 2016). Adenosine A_{2A} and A_{2B} receptors are both shown to couple G_s proteins and these go on to stimulate adenylate cyclase and further cause cAMP activation (Germack and Dickenson 2004; Borea et al. 2016). Adenosine A_3 receptors are also coupled to G_i, G_o and G_q proteins, similar to A_1 adenosine receptors, which ensures the inhibition of adenylate cyclase activity is achieved. A_3 adenosine receptors are also implicated in the activation of phospholipase C and D (Figure 1.4) (Germack and Dickenson 2004; Borea et al. 2014).

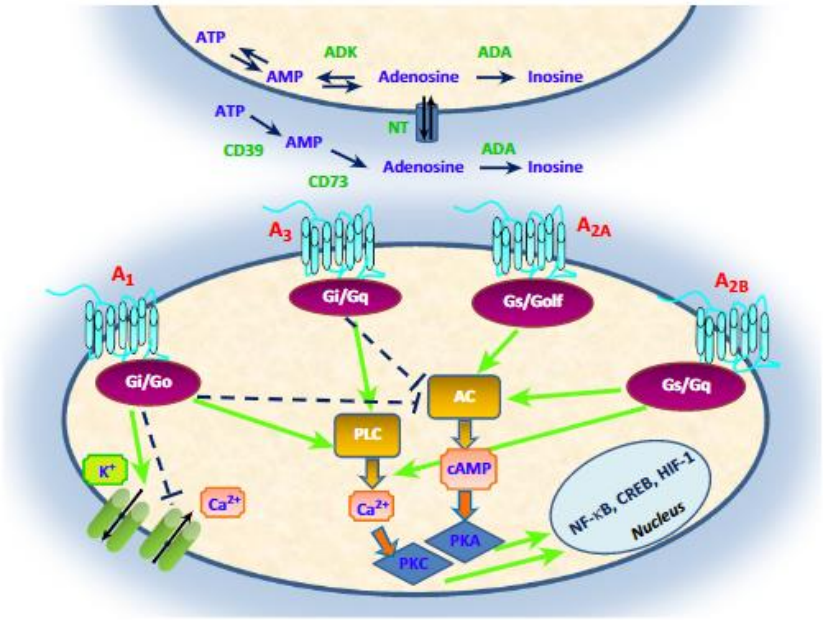


Figure 1.5 Schematic diagram of adenosine metabolism mediated through adenosine receptors (Boreo et al. 2016). Diagram illustrates an overview of adenosine biosynthesis and degradation as well as the second messenger pathways that are coupled to the four adenosine receptors.

Adenosine receptors are shown to be expressed within the cardiovascular system which includes the coronary artery, pulmonary artery, aorta and smoother muscle cells; but most importantly, adenosine receptors are expressed within cardiomyocytes that make up the myocardium (Sheth et al. 2014). Adenosine has the ability to cause vasoconstriction and vasodilation (Sheth et al. 2014). Not only are adenosine receptors beneficial for the cardiovascular system; they are also activated within the central nervous system to allow for neurotransmitter release (Cunha 2001; Sheth et al. 2014) and to regulate T-cell proliferation as well as cytokine production to ensure an inflammatory response can be mediated (Hasko et al. 2008; Sheth et al. 2014).

1.16 Adenosine

Adenosine itself is an endogenous nucleoside modulator that is released from almost all cells and it is generated by the dephosphorylation of ATP into extracellular AMP and then further broken down into adenosine (shown in Figure 1.5 above) (Gessi et al. 2011; Boreo et al. 2016). This nucleoside mediated through the activation of the four different adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) that are mediated through GPCRs. There is an estimated level adenosine

within the interstitial fluid in the range between 20-200nM (Fredholm 2011). The concentration of adenosine can increase when metabolically unfavourable conditions occurs such as tissue hypoxia (Fredholm 2010). This can lead to an enhanced breakdown in ATP to form more interstitial adenosine in an attempt to protect tissue from damage (Gessi et al. 2011). Adenosine levels can rise significantly above basal levels with different physiological conditions such as asthma, chronic inflammation, neurodegenerative disorders and cancer (Gessi et al. 2011). With an increase in adenosine levels, this can play a pivotal role in ischaemic injury and can further reduce the damage that occurs from a hypoxic event such as ischaemia (Gessi et al. 2011; Gaudry et al. 2020; Effendi et al. 2020).

Adenosine receptors are widely expressed in human organs and tissue spanning from the brain, heart, lungs, liver, bone, kidney, joints and blood cells (Peleli et al. 2017). Studies have also been conducted where all four adenosine receptors have been cloned and pharmacologically characterised within different specie groups (Gessi et al. 2011). It was found that the amino acid composition of adenosine receptors between human and rat species was 80-95% similar with more variance within the composition of the A_3 receptors amongst the other receptors (Ballesteros-Yáñez et al. 2018). In rat, the A_1 adenosine receptor has shown to be widely expressed within the brain, heart, liver, kidney and aorta (Dixon et al. 1996; Gessi et al. 2011). Within this same specie group, A_3 receptors are widely expressed within the heart and the central nervous system (Gessi et al. 2011). The A_{2A} adenosine receptors were shown to be expressed within the lungs, heart, kidney and brain and the A_{2B} receptors were researched to be expressed within the bowel, bladder, lungs and the brain (Dixon et al. 1996; Gessi et al. 2011).

Adenosine plays a direct role upon the cardiovascular system in terms of regulating blood flow, heart rate and pressure and studies have been carried out to determine the effects of certain receptor specific agonists on the various adenosine receptors. Activation of the A_1 adenosine receptor has been found to decrease heart rate, a study by Albrecht-Kupper, Leineweber and Nell (2012) suggested how A_1 agonist 2-Chloro-N^6-cyclopentyladenosine (CCPA) can significantly reduce heart rate in a concentration-dependent manner. Previous research conducted by Hoffman et al. (1997) showed that when A_1AR agonist N6-cyclopentyladenosine was administered to mammalian embryos it significantly decreased heart rate. This group also showed that there was no significant effect upon heart rate via the

A_{2A} and A_{2B} adenosine receptors however studies by Berwick et al. (2011) in anesthetized dogs and Sanjani et al. (2011) in knockout mice both showed that activation of the A_{2A} and A_{2B} receptors enhanced vasodilation and therefore increase coronary blood flow. Activation of A_3 adenosine receptors in mammalian embryos were shown to exert a mild decrease in heart rate (Sanjani et al. 2011). Activation of A_3 adenosine receptors within different species are shown to have different effects upon haemodynamic parameters. Maddock et al. (2002) showed that A_3AR agonist 2-CL-IB-MECA caused vasodilation and therefore increase coronary flow at concentrations of 100nM within rat heart. This is contrasting to earlier studies by Lasley et al. (1999) where it was reported that A_3AR agonists IB-MECA and CL-IB-MECA had no effect upon cardiac function with rat and rabbit with a further zero effect upon coronary flow in rabbits.

Research is being conducted into non-adenosine receptor drugs activating adenosine-related mechanisms. Some of these putative modulators include opiods, sildenafil, ketamine and creatine (Jacobson and Reitman 2020).

1.17 Cardioprotection

Several animal models including rat and human cardiomyocytes have demonstrated endogenous protective mechanisms to be activated. Ischaemic preconditioning is a mechanism of which repeated short cycles of ischaemia (mechanical occlusion) and immediate reperfusion are administered to the myocardium prior to the onset of ischaemia (Iliodromitis et al. 2007; Singh et al. 2018). Studies have shown that this method of cardioprotection has limited myocardial injury and has further provided the myocardium with a resistance to ischaemic insult (Skyschally et al. 2008; Tomai et al. 1999).Post-conditioning is when brief periods of ischaemia and reperfusion occur after the onset of reperfusion. Studies have shown post-conditioning to effectively protect the myocardium and further significantly decreasing infarct size (Skyschally et al. 2008). The viable endogenous protective mechanisms that can be activated through pre- and post-conditioning can include adenosine production, the opening of ATP-sensitive potassium channel as well as the release of nitric oxide (Verma et al. 2002; Singh et al. 2018; Covinhes et al. 2020; Gaudry et al. 2020).

1.18 Ischaemic Pre-conditioning

Ischaemic pre-conditioning was first discovered by Murray et al (1986) and research was shown to involve short episodes of non-lethal ischaemia which was able to delay necrosis upon cardiomyocytes and therefore prevent cell death. Further studies were carried out upon a canine model where 4 consistent periods of 5 minute coronary occlusions were shown to decrease infarct size by almost 75% when ultimately accompanied by 40 minutes of index ischaemia (Murray et al. 1986). Ischaemic pre-conditioning has been shown to confer cardioprotection through the reduction of infarct size within the swine model (Schott et al. 1991) and rabbit model (Liu et al. 1991); however further research has suggested that preconditioning has ultimately turned into a universal phenomenon where it can be observed in various organs and tissue, not only in the heart, but also in the liver, brain, retina, kidney, intestine and skeletal muscle (Yang et al. 2014; Clavien et al. 2000; Simon et al. 1993; Roth et al. 1998; Igarashi et al. 2013; Erling Junior et al. 2013; Addison et al. 2003; Stokfisz et al. 2017).

Ischaemic preconditioning has been researched to protect ischaemic reperfused myocardium through the release of endogenous agents that are locally released at the site of injury. These include; adenosine, bradykinin, catecholamine's and opioids that act upon the cell surface GPCRs (Cohen et al. 2000; Stokfisz et al. 2017). The activation via these mediators trigger an intracellular signal transduction where potentially three parallel pathways may activate (1) the mediation of nitric oxide (NO) and nitric oxide synthase (NOS), (2) the reperfusion injury salvage kinase (RISK) pathway and the (3) the survival activating factor enhancement (SAFE) pathway (Stokfisz et al. 2017). Yellon and colleagues (2002; 2003; 2004) were able to experimentally shown that ischaemic preconditioning has the ability to confer myocardial protection through the recruitment of the RISK pathway which consists of the PI3K-AKT and MEK1/2-ERK1/2 cell survival pathways and it was further shown that when these pathways were inhibited, there was also a halt in ischaemic pre-conditioning dependent cardioprotection. The activation of protein kinase C through ischaemic pre-conditioning was shown to elevate synthesis of various protective proteins and the opening of the ATP-sensitive K^+ channels (Tomai et al. 1999; Healy et al. 2015). One of the latest human clinical trials conducted by Candilo and colleagues (2015) showed that ischaemic pre-conditioning

showed a positive clinical benefit upon the heart in patients undergoing coronary artery bypass graft (CABG) and/or valve surgery (Candilo et al. 2015).

1.19 Pharmacological Pre-conditioning

The administration of pharmacological agents prior to ischaemia has experimentally shown to induce cardioprotection and this is a phenomenon referred to as pharmacological preconditioning (Yellon and Downey 2003). Downey and colleagues (2006) discovered that when acetylcholine was administered 30 minutes before ischaemia, there was a reduction in myocardial infarction damage within isolated rabbit hearts. This group also went on to study how protection of the myocardium involved the recruitment of the MEK1/2-ERK1/2 and PI3K-AKT cell survival pathway.

A recent study by Sampieri and colleagues (2020) showed that the administration of pharmacological agent Diazoxide to conduct pharmacological pre-conditioning in adult rat cardiomyocytes was shown to down-regulate store-operated Ca^{2+} channels which led to ensuring cardioprotection following an ischaemia-reperfusion insult (Sampieri et al. 2020).

Previous studies have elucidated how pharmacological pre-conditioning with adenosine agonist analogues have the ability to improve ischaemic ventricular dysfunction and when A_1AR antagonist was administered alongside, this protection was lost within the rat and rabbit species (Lasley et al. 1995). Pharmacological pre-conditioning with A_1AR agonist 2-chloro-N6-cyclopentyladenosine (CCPA) alongside lidocaine (which modulated sodium fast channels) had the ability to minimise myocardial damage in *in vivo* rat models (Canyon and Dobson 205). Pharmacological pre-conditioning with A_1AR agonist have further shown to mediate cardioprotection with rabbit (Tracy et al. 1998) as well as in the rat (Hochhauser et al. 2007). A study conducted by Safran et al. (2001) discovered that the administration of A_1AR agonist CCPA or A_3AR agonist CL-IB-MECA prior to ischaemia successfully attenuated cardiomyocyte injury and this protection was further abrogated when cardiomyocytes were pre-treated with A_1 antagonist DPCPX and A_3 antagonist MRS 1523, respectively. Pharmacological pre-conditioning with A_3AR agonists were shown to also mediate cardioprotection within rat (Hochhauser et al. 2007), mice (Zhao and Kukreja 2002) and rabbit (Takano et al. 2001). Previous research has suggested that pharmacological pre-conditioning with both A_1AR and

A_3AR agonists was shown to limit reperfusion injury within the human atrial model of ischaemia reperfusion injury (Carr et al. 1997).

Pharmacological pre-conditioning with A_2AR agonists have also potentially shown protection to ventricular myocytes from hypoxia/reoxygenation injury (Stickler et al. 2006).

1.20 Ischaemic Post-conditioning

Ischaemic post-conditioning is when brief episodes of ischaemia are applied to the myocardium at the early stages of reperfusion (Pagliaro and Penna 2011). This process has been suggested to be just as protective and effective as pre-conditioning against ischaemia-reperfusion injury. When post-conditioning is applied immediately within 30 minutes after the onset of reperfusion, this is referred to as rapid post-conditioning however if post-conditioning occurs hours or days after the onset of reperfusion then this can be considered as delayed post-conditioning (Zhou et al. 2009). Post-conditioning is known as brief cycles of ischaemia at the onset of reperfusion (Pagliaro and Penna 2011). Studies have been conducted in order to explore the effects of post-conditioning and Galagudza et al. (2004) assessed that post-conditioning limited infarct size and improved endothelial functioning within rat hearts that were subjected to ischaemia-reperfusion injury (Galagudza et al. 2004). Another study conducted by Zhou et al. (2003) discovered that post-conditioning decreased infarct size by 44% compared to the same protective effect of pre-conditioning in rat (Zhou et al. 2003).

Post-conditioning has been researched to be a powerful protective process however the definite intracellular signalling pathways that are activated in order to mediate protection remain to be fully explored (Zhao and Vinten-Johansen 2006). It was reported by Kin et al. (2005) that post-conditioning can protect the myocardium via the delayed washout of intravascular adenosine in mice. This was further studied and the administration of non-specific adenosine antagonist 8-SPT abolished any cardioprotective effects meaning that the cardioprotection detected when post-conditioning is carried out to the myocardium implicating a role for adenosine receptors in mediating cardioprotection (Kin et al. 2005).

Darling et al. (2005) also discovered that post-conditioning can exert its cardioprotective effects via the upregulation of the MEK1/2-ERK1/2 cell survival pathway, where this

protection was then blocked by MEK1/2 inhibitor PD98059 (Darling et al. 2005). Similar results were also studied by Yang et al. (2004) where they also discovered that the acquired protection through the opening of mitochondrial K_{ATP} channels was independent of the PI3K-AKT cell survival pathway (Yang et al. 2004). Further studies also went on to contradict previous studies where Yellon and colleagues (2004) discovered that when isolated perfused rat hearts underwent ischaemic post-conditioning, protection was acquired by the recruitment of the PI3K-AKT-eNOS cell survival pathway; therefore it is feasible to suggest that the exact role of the PI3K-AKT cell survival pathway in cardioprotection via post-conditioning remains elusive.

Current research has provided a sound basis for the processes of pre-conditioning and post-conditioning and that it can protect the ischaemic reperfused myocardium from ischaemia reperfusion injury within many species (Zhoa and Vinten-Johansen 2006) as well as human trials (Straat et al. 2003). The study by Straat et al. (2003) showed that when ischaemic post-conditioning reduced infarct size by 36% in patients that were admitted for coronary angioplasty. This study did show promising preliminary results for the application of ischaemic post-conditioning within a clinical setting; however further studies by Lasley and colleagues (2005) showed that post-conditioning failed to protect the human heart from ischaemia-reperfusion injury but they did show that post-conditioning was able to improve the blood perfusion to the myocardium.

1.21 Cardioprotection conferred by the activation of adenosine receptors at reperfusion

To date, numerous studies have shown the implication of adenosine to protect the myocardium from ischaemia-reperfusion injury. Zhao et al. (1993) conducted a classic investigation that demonstrated that when the A_1, A_2 and A_3 adenosine receptors were blocked by the non-selective adenosine antagonist 8-(p-sulfophenyl)-theophylline (8-SPT) at the onset of reperfusion. This limited endogenous adenosine mediated cardioprotection due to the blockade of the receptors suggested that endogenous adenosine played an important role to enhance cardioprotection (Yao et al. 1993; Covinhes et al. 2020).

Zhao et al. (2001) further went on to investigate the administration of adenosine at reperfusion to significantly limit infarct development in a series of experiments performed in

dogs. They concluded that the limitation of ischaemia reperfusion injury by adenosine was mediated by the upregulation of the anti-apoptotic protein Bcl-2 and further downregulated by pro-apoptotic protein Bax. There was also a significant decrease in necrosis discovered in the myocardium (Zhao et al. 2001).

Previously, investigators have attempted to implicate the role of adenosine either by pre-conditioning or administration at reperfusion and Heide et al. (1996) failed to observe protection administered at reperfusion in a canine model and failed to observe protection when adenosine was administered before coronary artery occlusion at reperfusion within a rabbit model of ischaemia-reperfusion injury (Goto et al. 1991).

Earlier studies focused on the A_1AR in terms of cardioprotection due to the lack of identification of the A_3ARs. Yao and Gross (1993) discovered that when A_1AR agonist cyclopentyladenosine (CPA) was administered at by the initial coronary artery occlusion, cardioprotection was observed (Yao and Gross 1993). Similar findings were also observed by Laing and Jacobson (1998). Lozza at al. (1997) showed that the adenosine A_1 receptor subtype and the A_{2A} receptors both mediated beneficial properties during ischaemia and reperfusion in isolated rat hearts even though the mechanisms of protection were not fully elucidated (Lozza et al. 1997). Stambaugh et al. (1997) also investigated the novel cardioprotective function of both the A_1 and A_3 adenosine receptors when undergoing hypoxia within chick embryos how they had the ability to attenuate myocyte injury (Stambaugh et al. 1997). Similar findings were investigated by Safran et al. (2001) where highly selective A_1AR agonist, CCPA and A_3AR agonist CL-IB-MECA both produced cardioprotective effects against ischaemia within isolated cardiac myocytes of new born rats (Safran et al. 2001).

More recent research showed that the mixed A_1/A_{2A} adenosine agonist AMP579 when given at reperfusion was protective within animal models of ischaemia-reperfusion injury. A further study was conducted to show that AMP579 was neither an A_1 nor A_{2A} selective agonist however it was then determined to be a potent A_{2B} agonist that also had the ability to protect myocardium against infarction through the A_{2B} adenosine receptor (Liu et al. 2010).

Baltos et al. (2017) have recently conducted a clinical trial on a partial A_1AR agonist, Capadenoson, which also has the ability to stimulate the A_{2B}AR. It was suggested that Capadenoson was a dual A_1/A_{2B} adenosine receptor agonist, however it did promote

cardioprotection and modulate a decrease in cardiac fibrosis in heart disease (Baltos et al. 2017).

Ample amounts of current research has been conducted upon the A_3 adenosine receptor agonist due to their characteristics being more well known compared to A_1 adenosine receptor agonists. Maddock et al. (2002) used a highly specific A_3AR agonist IB-MECA administered at reperfusion and concluded that it protected the myocardium from ischaemia-reperfusion injury and furthermore limited myocardial stunning in the guinea pig model. This protection that was acquired was blocked in the presence of A_3AR antagonist MRS1191 (Maddock et al. 2002) Maddock et al. (2003) also went on to show that another A_3AR agonist, 2-CL-IBMECA, when administered at reperfusion in isolated perfused rat heart as well as the cardiomyocyte model of ischaemia reperfusion injury, was able to confer cardioprotection. This protection was mediated through the anti-apoptotic and anti-necrotic manner and this protection was abrogated in the presence of A_3AR antagonist MRS1191 (Maddock et al. 2003). It would be feasible to suggest that research into A_1AR agonists could potentially confer cardioprotection?

Furthermore, research conducted by Hussain and colleagues (2013) also described the molecular mechanisms that were associated with A_3AR mediated cardioprotection and they indicated that within rat cardiomyocytes, the A_3AR agonist 2-CL-IB-MECA (1nM) was able to upregulate pro-survival signalling pathways that could lead to a decrease in caspase-3 activity to ameliorate ischaemia-reperfusion injury (Hussain et al. 2013).

Activation of A_3ARs have shown to confer cardioprotection within different models however these receptors have also been able to stimulate anti-cancer properties (Fishman et al. 2001); as well as played a role in cerebral-protection (Von Lubitz et al. 2001); as well as within anti-apoptotic and anti-necrotic pathways (Maddock et al. 2003). Studies have also shown that A_3AR agonists have protected the lungs against ischaemia reperfusion injury (Matot et al. 2006).

1.22 Cardioprotection conferred by the activation of adenosine receptors post-reperfusion

Previous studies elucidated how pharmacological and non-pharmacological interventions can protect the myocardium from ischaemia-reperfusion injury, however it remains an area for further research to establish whether postponing the administration of pharmacological agents after the onset of reperfusion has the ability to protect the myocardium from ischaemia reperfusion injury. Von Lubitz and colleagues (2001) investigated that when the administrations of A_3AR agonist IB-MECA was postponed to 20 minutes after the onset of reperfusion there was significant protection observed to the brains of mice that were subjected to ischaemia-reperfusion. The specific signalling pathway that was recruited was not determined (Von Lubitz et al. 2001). Another study conducted by Jonassen et al. (2001) investigated that when the administration of the hormone insulin occurred 15 minutes post-reperfusion, a loss of protection was observed in rat (Jonassen et al. 2000). Bolli et al. (2006) further showed that the administration of cytokine therapy 4 hours after the onset of reperfusion in order to limit ventricular modelling showed to improve left ventricular performance by the promotion of cardiac regeneration within mouse. Caspase inhibitors were also researched by Armstrong et al. (2001) that when administered 1 hour after reperfusion, they were able to reduce infarct development within the rat heart (Armstrong et al. 2001). Research by Hussain and colleagues (2015) also looked into how activation of the A_3 adenosine receptors at the onset of reperfusion/reoxygenation with the use of 2-CL-IB-MECA in rat perfused hearts and cardiomyocytes successfully decreased caspase-3 activity by the upregulation of pro-survival signalling pathways which included the MEK1/2-ERK1/2 and PI3K-AKT cell survival pathways (Hussain et al. 2015).

The following study may not be related to the myocardium however it does explore post-reperfusion activation within cerebral ischaemia of brain endothelial cells using two mouse models. The two models consisted of; global and focal cerebral ischaemia induced by transient occlusion of the carotid arteries, or the middle cerebral artery. The study conducted by Yu et al. (2017) investigated how cerebral ischaemia leads to multifaceted injury within the brain. This study showed that when a poly-therapeutic drug (lamotrigine and lovastatin) was administered after the onset of reperfusion, an increase in protection was observed via the targeting of multiple deleterious cascades (Yu et al. 2017).

Interestingly, the A_3AR agonist 2-CL-IB-MECA has previously been shown to limit infarct development within ischaemic reperfused hearts and it has also been shown that 2-CL-IB-MECA has the ability to mediate cardioprotection when administered at reperfusion and post-reperfusion via the recruitment of the PI3K-AKT and MEK1/2-ERK1/2 cell survival pathways (Hussain 2009). Hussain et al. (2013) further demonstrated that the recruitment of the PI3K-AKT and MEK1/2-ERK1/2 cell survival pathways was abolished when kinase inhibitors Wortmannin and UO126 (respectively) were administered. They also showed that when A_3AR agonist 2-CL-IB-MECA was administered throughout reoxygenation, there was a significant reduction of apoptosis, necrosis, cleaved-caspase 3 activity as well as an increase in BAD expression in rat cardiomyocytes (Hussain et al. 2013).

Collectively, research has tended to show adenosine to confer cardioprotection when administered at reperfusion or prior to an ischaemic insult however, no study to date has shown the effect of an A_1AR agonist being administered post-reperfusion upon infarct development as well as upon anti-apoptotic and anti-necrotic processes. No clear link between A_1 adenosine receptors and the recruitment of cell survival pathways has been shown via the delayed reperfusion activation of A_1ARs. The aim of this current study was to determine whether A_1AR agonist 2'-MeCCPA mediated cardioprotection within the Langendorff perfused rat heat and adult rat cardiomyocyte model from reperfusion or reoxygenation injury via the recruitment of the PI3K-AKT and MEK1/2-ERK1/2 cell survival pathways post-reperfusion/reoxygenation.

1.23 Reperfusion Injury Signalling Kinase (RISK) Pathway

Studies by Hausenloy et al. (2005) have previously established the role and recruitment of pro-survival kinases; AKT and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2). These kinases are commonly termed the RISK pathway and have shown to confer cardioprotection against myocardial ischaemia-reperfusion injury. This makes these pro-survival kinases an important target for cardioprotection. Further studies have also been conducted to show that the RISK pathway involves the activation of PI3K-AKT or ERK1/2 as two parallel branches of the RISK pathway (Hausenloy et al. 2007). The PI3K-AKT and ERK1/2 signalling pathways are not independent of each other as a study conducted by Hausenloy et

al. (2004) showed that isolated perfused rat hearts that were subjected to LY294008 (PI3K-AKT inhibitor) at reperfusion induced phosphorylation of ERK1/2-p70S6K; furthermore inhibiting MEK1/2-ERK1/2 pathway using PD98059 at reperfusion induced phosphorylation of AKT. Therefore this suggests cross talk between the two kinase pathways (Hausenloy and Yellon 2004).

1.23.1 Mitogen-Activated Protein Kinase (MAPK)

Mitogen activated protein kinases (MAPK) are a family of kinases that have the ability to respond to extracellular stimuli. MAPKs are serine and threonine kinases and that have been investigated by a number of research groups and found to regulate cell growth, cell differentiation, apoptosis and stress responses (Figure 1.6) (Plotnikov et al. 2011). Three MAPK families have been clearly characterised and researched, namely, the classic MAPK known as ERK, C-Jun C-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 kinase. MAPKs lie within protein kinase cascades (Zhang and Liu 2002). Kinases can be activated by tyrosine kinase receptors of the activation of GPCRs couple with G_i, G_o, G_q and G_s (Pearson et al. 2001).

Once receptor ligand interaction occurs, a sequence of events involving adapter proteins, growth factor receptor binding protein (GRB) and Ras guanine exchange factor (SOS), can activate Raf (an MAP kinase kinase kinase (MAPKKK), which is also the first of the three MAPK module as seen in Figure 1.5 (Zhang and Liu 2002). The phosphorylated Raf has the ability to activate downstream MAPKK, MEK1/2 and can further phosphorylate to produce MAPK, ERK1/2. The phosphorylation of ERK1/2 at Thr_{202} and Tyr_{204} residues can transform ERK1/2 into its active form (Wechsler et al. 1994; Zhang and Liu 2002).

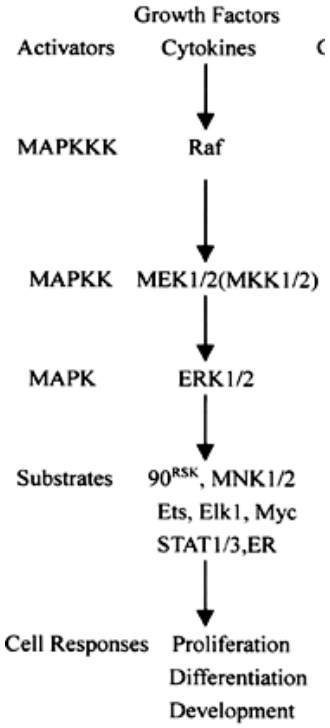


Figure 1.6 Diagram to illustrate the MAPK cascade to produce MEK1/2 and ERK1/2 (Zhang and Liu 2002).

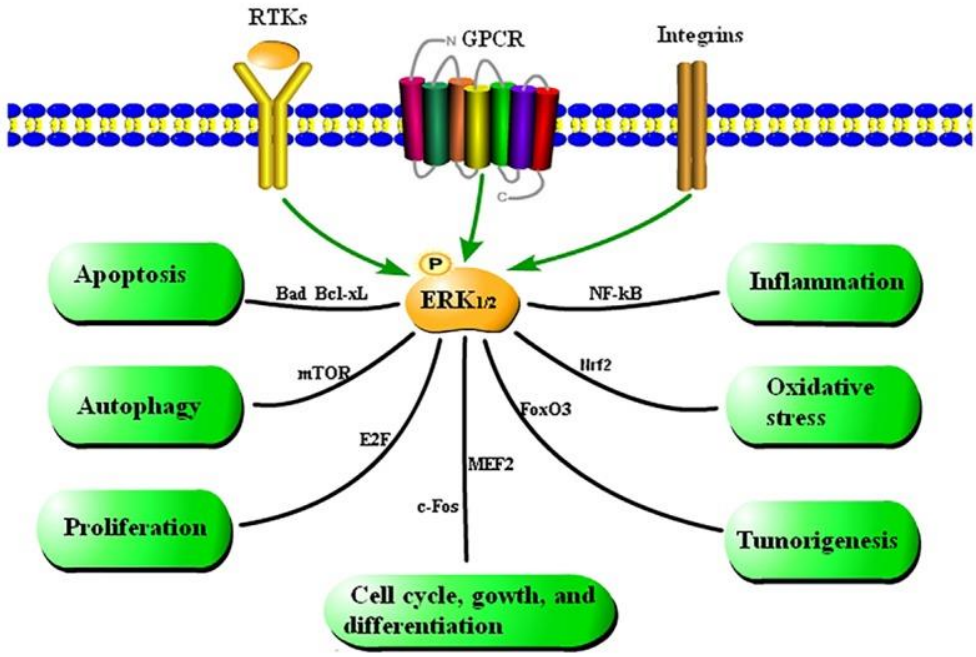


Figure 1.7 Schematic diagram to summarise the functions of the ERK1/2 signalling pathway (Kong et al. 2019).

Punn et al. (2000) have shown that an increase in ERK1/2 during ischaemia can further be upregulated at the onset of reperfusion within cardiomyocytes. Later studies by Schulte et al. (2002) have characterised adenosine receptor agonists to cause the upregulation of extracellular regulated kinase (ERK1/2) within Chinese hamster ovary cells (CHO) that expressed A_3 adenosine receptors.

Germack and Dickenson (2004) investigated the characteristics of the activation of ERK1/2 via adenosine and adenosine analogues within new born cardiomyocytes. They characterised that when A_1, A_{2A} and A_3 adenosine receptor analogues were administered in a time and dose dependent manner, there was an increase in ERK1/2 activation however this was not to the same extent as with adenosine. This suggests that adenosine has the ability to stimulate all adenosine receptors however specific receptor agonists exert their effect on their respective receptor subtypes. Nevertheless, a link between adenosine and the upregulation of ERK1/2 has been investigated.

Hussain et al. (2013) investigated the role of the MEK1/2-ERK1/2 signalling pathway and the effect it had on modifying caspase-3 activity by A_3AR agonist 2-CL-IB-MECA mediated cardioprotection in rat cardiomyocyte model. It was shown that caspase-3 activity was decreased via the recruitment of the MEK1/2-ERK1/2 pathway and MEK1/2 inhibitor UO126 abrogated this mediation of cardioprotection (Hussain et al. 2014).

The stress activated protein kinases p38 and JNK are more responsive to a stress stimuli such as heat shock, UV radiation, osmotic shock and they also play a part in cell death. Studies have been conducted where the inhibition of p38 was shown to reduce myocardial injury following infarction within mouse however this protection was not seen in swine (Kaiser et al. 2005). JNK has remained a pathway of disagreement on whether it is anti- or pro-apoptotic as recent study by Xu et al. (2014) has shown that inhibiting JNK dephosphorylation and activating ERK1/2 phosphorylation can cause protection against myocardial ischaemia-reperfusion injury.

1.23.2 Phosphatidylinositol-3-Kinase (PI3K)

Phosphatidylinositol-3-kinase (PI3K) is a serine/threonine kinase which is a significant player in the role of regulating cell growth, differentiation and survival (Ban et al. 2008). The PI3K-AKT pathway itself has been shown to catalyse the phosphorylation of inositol-containing lipids, also known as phosphatidylinositol's which are then converted to phosphatidylinositol-3, 4, 5-trisphosphate which are important second messenger molecules. The PI3K phosphorylation can lead to subsequent phosphorylation of AKT (also known as protein kinase B) via cell signalling cascades which can further lead to cardioprotective effects in a myocardial ischaemia-reperfusion setting (Jonassen et al. 2004; Mangi et al. 2003). This cell survival pathway was also researched to investigate whether it plays a role within a pre-conditioning setting (Hausenloy and Yellon 2003). The phosphorylation of AKT via the cell signalling cascade leads onto the dual phosphorylation of the ser_{473} and thr_{308} residues by phosphate-dependent kinase-1 (PDK-1) (Ban et al. 2008).

The activation of the PI3K-AKT pathway can promote cell survival by the phosphorylation of various pro survival molecules such as AKT, endothelial nitric oxide synthase (eNOS) and p70S6K (Hausenloy et al. 2004; Mocanu and Yellon 2002). These studies have demonstrated a decrease in the activation of apoptotic proteins such as p53, caspase and members of the Bcl-2 family (BIM, BAD and BAX). AKT can activate a variety of effector proteins such as glycogen synthase kinase 3β (GSK3β) which can stimulate glycogen synthesis as well as other proteins involved in cell proliferation and growth (Hausenloy and Yellon 2004; Park et al. 2006).

When exploring the context of myocardial ischaemia and reperfusion, studies were carried out by Punn et al. (2000) on cardiomyocytes models to simulate ischaemia and reperfusion in order to characterise the expression of AKT. It was shown that during ischaemia, p-$AKT_{(ser473)}$ was not expressed however expression was enhanced by reperfusion. The PI3K inhibitor Wortmannin was also able to abrogate AKT phosphorylation at reperfusion.

The administration of non-selective adenosine agonist NECA was shown to upregulate phosphorylation of AKT and the PI3K inhibitor Wortmannin or LY294002 abrogated the phosphorylation of AKT mediated by NECA in rabbit hearts (Yang et al. 2004). The same researchers further showed that the phosphorylation of PI3K was sensitive to the $G_{i/o}$ inhibitor

pertussis toxin which therefore implicates the activation of A_3 adenosine receptors by NECA as well as activation of MAPK p38 and ERK1/2. Kis et al. (2003) also showed the upregulation of PDK1-PI3K-AKT cell survival pathway within the rabbit model of ischaemic pre-conditioning to allow for protection of the myocardium.

A more recent study by Hussain et al. (2013) has proposed the recruitment of the PI3K-AKT cell survival pathway to enhance cardioprotection by decreasing capase-3 activity through the administration of A_3AR agonist 2-CL-IB-MECA at the onset of reperfusion in rat cardiomyocyte model. This highlight's the importance of the activation of the PI3K-AKT cell survival pathway which mediates protective effects to the myocardium.

Tian et al. (2015) demonstrated that pre-treatment to the myocardium with A_{2B}AR agonist BAY 60-6583 via pre-conditioning had the ability to protect the myocardium from ischaemia-reperfusion injury through its anti-inflammatory effects that also linked to the PI3K-AKT cell survival pathway.

Hausenloy et al. (2004) previously proposed the strategy of cross talk between the PI3K and the ERK1/2 pathways during early reperfusion. This novel study investigated how the blockage of the PI3K activity using PI3K inhibitor Wortmannin upregulated ERK1/2 phosphorylation and the blockage ERK1/2 pathway using PD98059 upregulated the AKT phosphorylation. Blockage of both pathways abrogated any protective effect by ischaemic pre-conditioning.

As a collective, data has demonstrated that the release of adenosine during myocardial ischaemia and reperfusion plays a punitive role in the activation of adenosine receptor dependent cell survival pathways (Sommerchild and Kirkeboen 2000; Hussain et al. 2013). Studies have looked into cell lines and rat cardiomyocytes and evidence is apparent to suggest that adenosine has the ability to activate AKT and ERK1/2 via adenosine receptors. There has been extensive research over the year's entailed pharmacological and non-pharmacological experimental interventions in order to protect the myocardium from ischaemia-reperfusion injury. Continued research in the field of cardiovascular research and the continuous development of agents could potentially lead to an agent that can one day be successfully used in a clinical setting.

1.24 Aims, Objectives and Hypotheses

1.24.1 Aims

The aims of this thesis were to:

- To determine whether the activation of A_1AR agonist 2'-MeCCPA, when administered at reperfusion or post-reperfusion had the ability to protect the myocardium from ischaemia-reperfusion injury via the PI3K-AKT or MEK1/2-ERK1/2 cell survival pathways within isolated perfused rat heart.
- To elucidate the status of cell survival pathway proteins AKT within non-treated control ischaemic-reperfused hearts and 2'-MeCCPA treated hearts when 2'-MeCCPA was administered at the onset of reperfusion or post-reperfusion in the presence and absence of their respective inhibitors by western blot analysis.
- To fully determine whether the administration of 2'-MeCCPA at the onset of reoxygenation or post-reoxygenation can attenuate cell death in the form of caspase-3 activity, apoptosis and necrosis in an adult rat cardiomyocyte model of hypoxia/reoxygenation injury. To also determine the role of the PI3K-AKT and MEK1/2-ERK1/2 cell survival pathways within 2'-MeCCPA mediated cardioprotection within the rat cardiomyocyte model.

1.24.2 Objectives

The objectives of this thesis were to:

- Examine the effects of increasing concentrations of 2'-MeCCPA in an ischaemia/reperfusion setting in isolated rat hearts as well as adult rat cardiomyocytes;
- Identify the concentration of the A_1AR agonist 2'-MeCCPA that provides the myocardium protection in the model of ischaemia/reperfusion and respectively hypoxia/reoxygenation;

- Investigating the effects of administrating 2'-MeCCPA at the onset of reperfusion, 15 or 30 minutes post-reperfusion to explore the protective effects.
- Investigate the effects of A_1 adenosine antagonism to confirm the protective role of the A_1 adenosine receptor in the model of ischaemia/reperfusion and hypoxia/reoxygenation, at the onset of reperfusion/reoxygenation, 15 and 30 minutes post-reperfusion/reoxygenation;
- Investigate the effects of 2'-MeCCPA on the PI3K-AKT intracellular signalling pathway in heart tissue, at the onset of reperfusion/reoxygenation, 15 and 30 minutes post-reperfusion/reoxygenation;
- Investigate the effects of 2'-MeCCPA on the MEK1/2-ERK1/2 intracellular signalling pathway in heart tissue, at the onset of reperfusion/reoxygenation, 15 and 30 minutes post-reperfusion/reoxygenation;

1.24.3 Hypotheses

The hypotheses of this thesis are as follows:

- The administrations of the A_1AR agonist 2'-MeCCPA will provide a cardioprotective effects to the myocardium in the ischaemia/reperfusion and hypoxia/reoxygenation model.
- This cardioprotective effect will be observed by a decrease in infarct size, an improvement to cardiac functioning, and a decrease in cell death and caspase-3 activity and an increase in the PI3K and MEK1/2 survival pathways at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion.

Chapter 2: Materials and Methods

2.1 Experimental Animals

Adult male Sprague Dawley rats (350±50g body weight) were obtained within this study from Charles River (Margate, UK) and were kept within the institutional animal house under humane conditions in order to acclimatise with free access to a standard pelleted diet and water. All care and procedures were carried out in accordance with the Guidance on the Operation of the Animals Act (Scientific Procedures Act 1986). Ethical approval was obtained from Coventry University Research ethics committee in order for the study to be carried out; ethics was regularly assessed throughout the project (Reference P76726).

2.2 Materials

The following chemicals 2-Chloro-*N*-cyclopentyl-2'-methyladenosine (2'-MeCCPA) (selective A_1 agonist), 1S,6bR,9aS,11R,11bR)11-(Acetyloxy)1,6b,7,8,9a,10,11,11b-octahydro-1-(methoxymethyl)-9a,11b-dimethyl-3H-furo[4,3,2de]indeno[4,5,-h]-2-h]-2-benzopyran-3,6,9-trione (Wortmannin) (selective irreversible PI3K inhibitor), 1,4-Diamino-2,3-dicyano-1,4-*bis*[2-aminophenylthio]butadiene (U0126) (selective inhibitor of MEK1/2), 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) (selective A_1 antagonist), 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) (highly selective A_{2a} antagonist), *N*-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide (MRS 1754) (selective A_{2b} antagonist), *N*-[2-(2-Furanyl)-8-propyl-8*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-yl]-*N*'-(4-methoxyphenyl)urea (MRE 3008F) (selective A_3 antagonist), 8-(p-Sulfophenyl)theophylline (8-SPT) (Unselective adenosine antagonist) and Hydrogen Peroxide were supplied supplied by Tocris (Bristol, UK).

All drugs were dissolved in dimethyl sulfoxide (DMSO) with the final concentration of DMSO being less than 0.02% as this does not affect haemodynamic data or infarct size (data not included) which is then stored at -20 °C. Bliksøen et al. (2016) investigated that when DMSO was administered under 0.02% to a Langendorff experiment, no effect was detected upon infarct size and haemodynamic parameters.

All salts and chemicals that were used in order to prepare buffers for the Langendorff technique and cardiomyocyte isolation were purchased from Fischer Scientific (Loughborough, UK).

All equipment and reagents used for the Western blot technique were purchased from Bio-Rad (Watford, UK).

All antibodies including: Cleaved Caspase-3 (Asp175) Antibody (Alexa Fluor® 488 Conjugate), phospho-AKT_{ser473}, total-AKT, phosphor-p44/42 MAPK $(ERK1/2)_{(Thr202/Try204)}$, p44/42 MAPK (ERK1/2) antibodies and GAPDH, (HRP) conjugated Rabbit monoclonal antibodies and antibiotin were purchased from Cell Signalling (UK). SuperSignal West Femto® enhanced chemiluminescent substrates were purchased from ThemoFisher (UK). Dead Cell Apoptosis Kit with Annexin V FITC and PI was purchased from ThermoFisher (UK).

2.3 Langendorff Technique – Isolated Perfused Rat Heart Model

2.3.1 Heart Extraction and Perfusion Procedure

Following the rapid sacrifice of the rat by cervical dislocation in accordance to the Schedule 1 Home Office Procedure, hearts were dissected and then placed in ice cold Krebs Henseleit buffer (KHB) solution (118.5mM NaCl, 25mM $NaHCO_3$, 4.8mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 12mM Glucose, 1.7mM $CaCl_2.2H_2O$) <4^oC and at pH 7.4 (Hussain et al. 2013).

The hearts were removed by a process called thoracotomy (Bell et al. 2011). This process involves a trans-abdominal skin incision is carried out at the xyphoid- sternum and further expanded to the lateral ends of the right and left costal sides (Bell et al. 2011). The surgical procedure was then continued up to the ribs at the left and right anterior axillary lines in order to generate the clamshell thoracotomy (Bell et al. 2011). Following on from this, the pericardium was opened and the thoracic cage was detached to expose the heart for dissection (Bell et al. 2011).

The hearts were removed by promptly excising the descending aorta and immediately transferred to ice cold KHB so that ischaemic damage was prevented (Skrzypiec-Spring et al. 2007). The aorta of the rat hearts were attached onto the aortic cannula by the aortic clip and perfused with KHB in a retrograded manner (Bell et al. 2011; Gharanei et al. 2013). The aorta was fastened to the aortic cannula by thread which secured the heart to the Langendorff system after which the aortic clip was removed. The KHB was administered retrogradely at a constant hydrostatic pressure, stable flow rate and at a constant pH of 7.4 by continuous gassing with 95% O_2 and 5% CO_2 (BOC gases). Extra care needed to be taken to ensure that the cannula was not inserted too deep into the aorta as this would cause mechanical fissure or obstruction to the coronary ostia (Bell et al. 2011). The temperature was also maintained at 37^{o}C with the use of a water jacketed heat exchange coil to allow for the heart to stabilise upon the Langendorff system (Skrzypiec-Spring et al. 2007; Bell et al. 2011). It has been researched that the optimal time needed for the heart to stabilise functioning is 5-10 minutes after the cannulation period (Skrzypiec-Spring et al. 2007; Bell et al. 2011).

The Langendorff model was first pioneered by Oscar Langendorff in 1895 and ever since evolved as a technique in which the understanding of the physiology of the heart which includes contractile function, coronary blood flow and cardiac metabolism could be understood (Bell et al. 2011). This model has been included within many research studies where the Langendorff model was used to probe and identify the pathophysiology of ischaemia and reperfusion as well as other disease states. This model can also study the effects of pharmacological agents and their physiological impact upon the heart as well as upon intracellular signalling pathways (Bell et al. 2011). Within a normal *in vivo* heart, it has been found that the coronary system relies upon the pressure within the heart in order to pump blood; however within a Langendorff system, the model itself pumps directly into the coronary system which then allows for a constant pressure within the heart. Whilst this is occurring, a steady ejection of KH buffer is maintained through the heart chambers. This way, pressure and flow is maintained as well as contractile activity (Bell et al. 2011; Cheung et al. 2000).

2.3.2 Measurement of Haemodynamic Cardiac Parameters: Left Ventricular Developed Pressure (LVDP), Heart Rate (HR) and Coronary Flow (CF)

A pressure transducer was calibrated prior to every Langendorff experiment using the software LabChart 7 with the PowerLab (AD Instruments Ltd. Chalgrove, UK). Before each experiment commenced, the pressure transducer was set to the zero point by the bridge amp and desired calibration values were applied and values were recorded in the software with applicable units (mmHg) for the whole experiment.

As soon as the heart contracted consistently, the left atrium was removed and a latex balloon was inserted into the left ventricle and inflated with water to a set preload of 5-10mmHg (Skrzypiec-Spring et al. 2007); Bell et al. 2011). The balloon was connected to a pressure transducer which allowed for the LVDP and HR to be recorded constantly and further examined through the PowerLab system (Figure 2.1) (Gharanei et al. 2013).

The haemodynamic parameters of LVDP and HR were observed and recorded at regular time intervals. To measure the CF rate, the effluent from the heart attached onto the Langendorff system was collected for 1 minute at each time point of regular intervals. All three of the haemodynamic parameters were recorded every 5 minutes in the stabilisation and ischaemia periods and every 15 minutes within the reperfusion period.

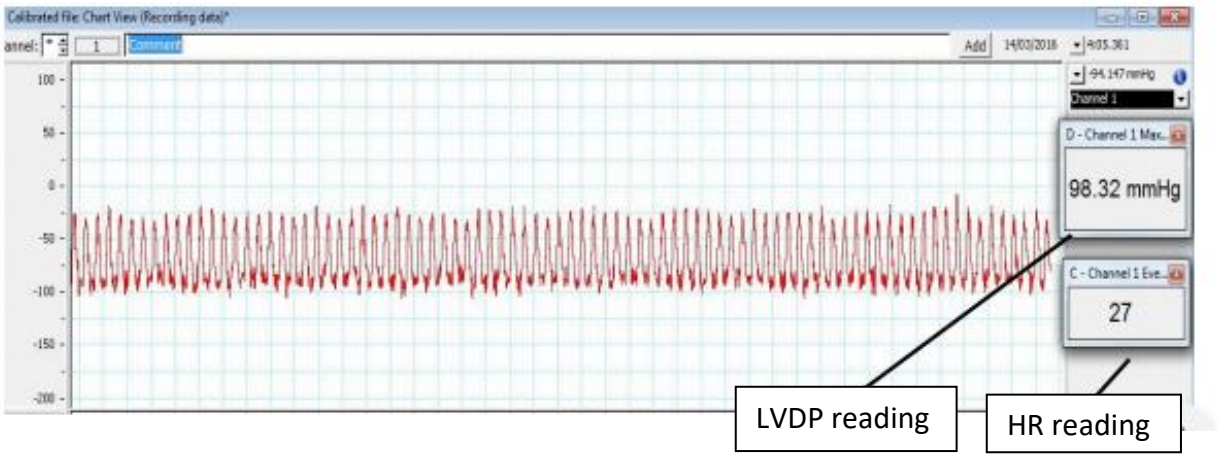


Figure 2. 1 Diagram illustrating a Langendorff trace that includes the heart rate and left ventricular developed pressure using the PowerLab system.

All haemodynamic parameters were graphed using Microsoft Excel where LVDP and HR were calculated as a percentage of the mean stabilisation period; whereas CF was adjusted to the heart weight and calculated as a percentage of the mean stabilisation period. Any hearts that had a high coronary flow rate (>20ml/min) or any arrhythmic episode throughout the 20 minutes to stabilisation period were excluded from the study.

2.3.4 Induction of Ischaemia

Regional ischaemia was induced by ligation where a surgical needle (also known as a suture) obstructed the main left coronary artery which further obstructed the flow to the left coronary artery. The occlusion point was established to be above the coronary artery branch. The ends of the suture were then passed through plastic tubes to form a tight snare to initiate ischaemia. Reperfusion was initiated by the removal of the plastic tubes to release the thread ends of the suture. After the completion of the reperfusion period, the surgical threads were tightened to obstruct the left coronary artery and a further 1ml of saline solution containing 0.25% Evans Blue was administered slowly through the aorta to delineate the viable areas from the infarct areas. Figure 2.2 shows a perfused rat heart before ischaemia, after ischaemia and then at the end of reperfusion when the heart is infused with Evans blue solution.

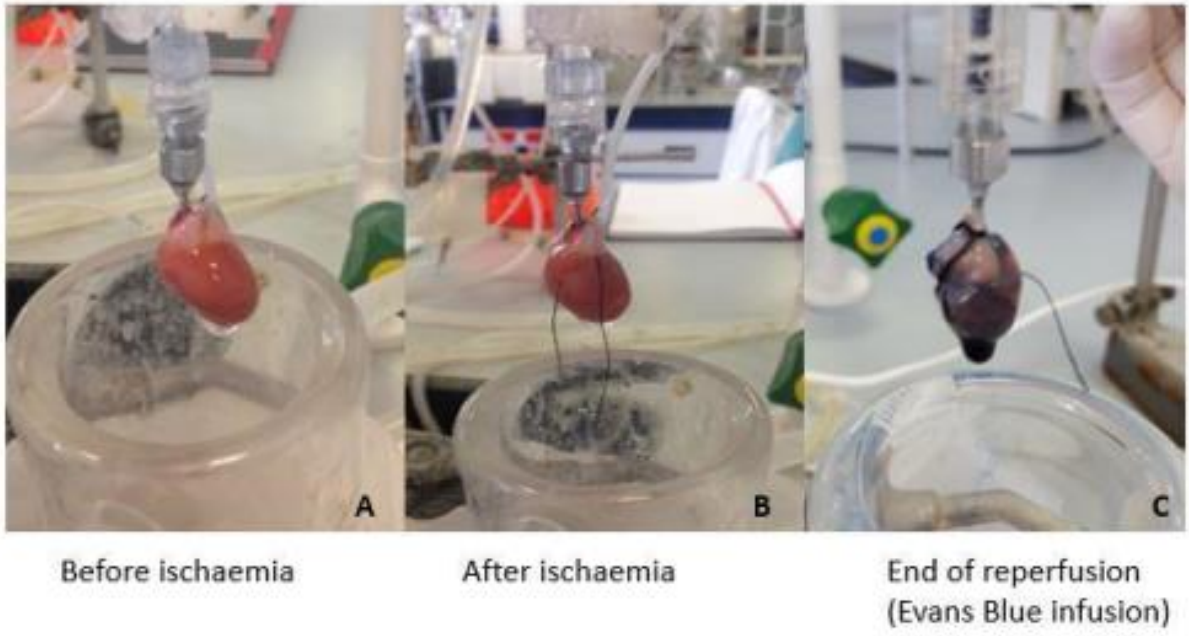


Figure 2. 2 The Langendorff setup illustrating a Sprague-Dawley rat heart throughout the stabilisation before ischaemia (A); regional ischaemia was induced by the tightening of a snare to ligate the left anterior descending artery (B); the end of reperfusion where the surgical thread was tightened and Evans blue was perfused through the aortic cannula (C)

2.3.5 Experimental Groups in Langendorff Protocol

Normoxic Control Group:

The normoxic control group consisted of the heart stabilising for 20 minutes followed by 155 minutes of perfusion with KHB as seen in Figure 2.3. Hearts were then weighed and stored at -20°C for triphenyltetrazolium chloride (TTC) staining.

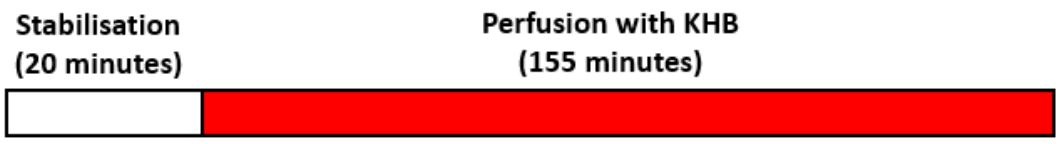


Figure 2. 3 Diagram showing the experimental protocol for isolated perfused rat hearts within the normoxic control group undergoing 20 minutes of stabilisation and 155 minutes of perfusion with KHB.

Ischaemia-Reperfusion (IR) Control Group:

Within the ischaemia/reperfusion (IR) control group, hearts underwent 20 minutes stabilisation, 35 minutes of induced regional ischaemia and a further 120 minutes of reperfusion as seem in Figure 2.4. On completion of reperfusion, surgical threads were tightened to obstruct the left coronary artery and a further 1ml of saline solution containing 0.25% Evans Blue was administered slowly through the aorta to delineate the viable areas from the infarct areas. Hearts were then weighed and stored at -20^{o}C for triphenyltetrazolium chloride (TTC) staining.

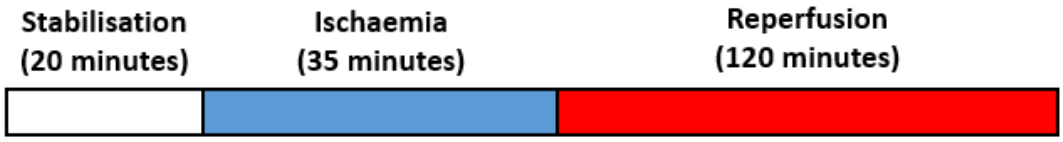


Figure 2. 4 Diagram showing the experimental protocol for isolated perfused rat hearts within the ischaemia/reperfusion (IR) control group undergoing 20 minutes of stabilisation, 35 minutes of induced regional ischaemia and a further 120 minutes of reperfusion with KHB.

Experimental Groups at Reperfusion Studies:

Hearts all underwent 20 minutes of stabilisation, 35 minutes of induced ischaemia and a further 120 minutes of reperfusion. As seen on Figure 2.5 at the onset of reperfusion, hearts were concomitantly infused with either:

a) KHB (IR control).
b) A_1AR agonist 2'-MeCCPA (0.1nM, 1nM, 10nM, 10nM and 1µM) (To determine cardioprotective effects of A_1AR agonist 2'-MeCCPa and 10nM was shown to be the most cardioprotective and used for further studies).
c) A_1AR agonist 2'-MeCCPA (10nM) in the presence of cell signalling cascade inhibitors – PI3K inhibitor Wortmannin (100nM); MEK1/2 inhibitor UO126 (10µM) (To determine the effects of 2'-MeCCPA (10nM) via the activity of the PI3K-AKT and MEK1/2-ERK1/2 cell signalling pathways).
d) A_1AR agonist 2'-MeCCPA (10nM) in the presence of selective A_1AR antagonist DPCPX (200nM) or unselective adenosine antagonist 8-SPT (1µM).

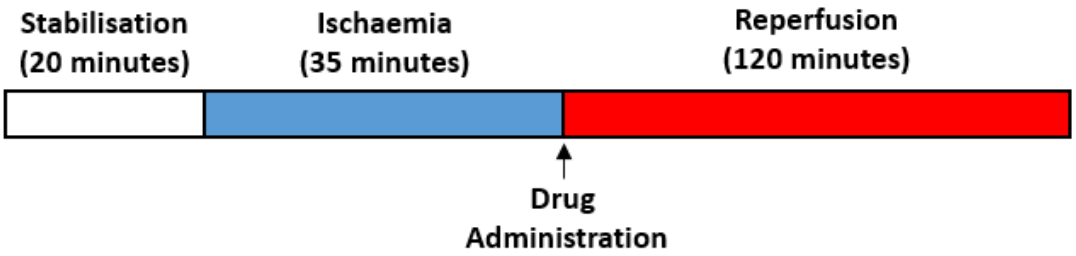


Figure 2. 5 Diagram showing the experimental protocol for isolated perfused rat hearts when subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes of reperfusion. Drug treatments were administered at the onset of reperfusion.

Upon completion of reperfusion, surgical threads were tightened to obstruct the left coronary artery and a further 1ml of saline solution containing 0.25% Evans Blue was administered slowly through the aorta to delineate the viable areas from the infarct areas. Hearts were then weighed and stored at -20^{o}C for triphenyltetrazolium chloride (TTC) staining.

Experimental Groups at Post-Reperfusion Studies:

As seen from Figure 2.6, hearts underwent 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes of reperfusion. Hearts were concomitantly infused with drug treatment at either 15 minutes or 30 minutes post-reperfusion with:

a) 2'-MeCCPA (10nM) at 15 or 30 minutes post-reperfusion.
b) 2'-MeCCPA (10nM) in the presence of PI3K inhibitor Wortmannin (100nM) or MEK1/2 inhibitor UO126 (10µM) at 15 of 30 minutes post-reperfusion.
c) 2'-MeCCPA (10nM) in the presence of either selective A_1 (DPCPX-200nM) or unselective 8-SPT (1µM) adenosine antagonists at 15 or 30 minutes post-reperfusion.

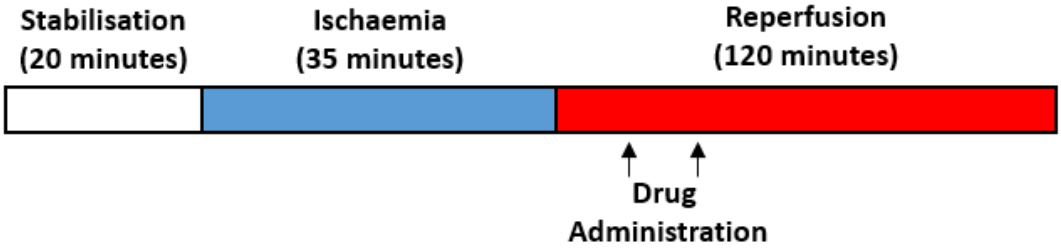


Figure 2. 6 Diagram showing the experimental protocol for isolated perfused rat hearts when subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes of reperfusion. Drug treatments were administered 15 or 30 minutes post-reperfusion.

Upon completion of reperfusion, surgical threads were tightened to obstruct the left coronary artery and a further 1ml of saline solution containing 0.25% Evans Blue was administered slowly through the aorta to delineate the viable areas from the infarct areas. Hearts were then weighed and stored at -20^{o}C for triphenyltetrazolium chloride (TTC) staining.

2.3.6 Triphenyltetrazolium Chloride Analysis (TTC)

At the end of the reperfusion period, all hearts including controls and experimental group hearts were de-cannulated and then weighed and frozen at -20^{o}C. Next, frozen hearts were cut in a transverse manner into slices of approximately 2mm thick and then incubated in triphenyltetrazolium chloride (TTC) solution (1% of TTC in phosphate buffer, pH 7.3 at 37^{o}C) for 10 minutes (Bell et al. 2011). This way of TTC staining is dependent upon the capacity of intracellular dehydrogenase enzymes that react with the tetrazolium salts to produce formazan (Bell et al. 2011). As a result of TTC staining, the viable cells which contained preserved NADPH were stained in a brick red colour. The non-viable cells (infarct cells) showed as pale white-yellow colour (Bell et al. 2011). Any non-risk areas were further stained dark blue from the Evans blue solution that was administered. Furthermore, the heart tissue slices were incubated in 10% formaldehyde for 4 hours prior to analysis. This allowed for distinct contrast between the infarct and risk areas.

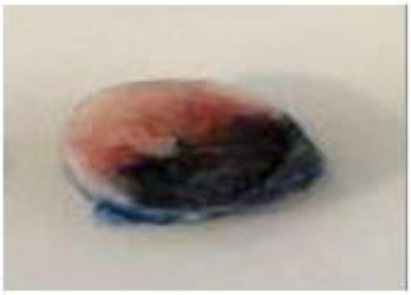


Figure 2. 7 TTC stained heart slice placed between two Perspex sheets that were compressed with the bulldog clips showing the left ventricle as the non-risk area (dark blue), viable risk area (brick-red) and infarcted area (pale white/yellow).

2.3.7 Infarct Size to Risk Ratio (%) Assessment

In order to assess the infarct size to risk ratio (%), the transversal heart slices were placed between two glass plates in order to expand the tissue slices and were then held together by clips. The viable area, non-risk area (dark blue), risk area (brick red) and infarct area (pale white yellow) (as represented in Figure 2.7) were then traced on an acetate paper with each trace then being examined using the ImageJ tool program (N.I.H Software, Bethesda, USA). The ratio of the infarct area within the risk area was evacuated for each heart slice. The viable, risk and infarct area within each heart slice was traced in a double blinded manner by an unconnected individual to ensure that there was no bias in results. A selection of samples were also double analysed to ensure all results were obtained in an accurate manner.

The infarct size (IS) for each slice within each group was calculated as a percentage of the area at risk (AAR) (IS/AAR %). The area at risk is the area free from Evans blue solution which corresponds to the myocardial perfusion bed distal to the occluded left anterior descending artery (Redford et al. 2012).

2.4 Adults Rat Ventricular Cardiomyocyte Isolation

Adult ventricular rat myocytes were isolated from male Sprague Dawley rats (350±50g body weight) via enzymatic dissociation (Maddock et al. 2002; Hussain et al. 2014). Once excision occurred, the hearts were place within ice cold KH buffer for a short period of time before being mounted upon the Langendorff apparatus and then perfused with modified calcium free Krebs buffer containing (in mM): 116 NaCl, 5.4 KCl, 0.4 $MgSO_4.7H_20$, 10 glucose, 20 taurine, 5 pyruvate, 0.9 Na_2HPO_4. $12H_2O$ and 25 $NaHCO_3$ dissolved in RO water. The buffer was further oxygenated with 95% O_2 and 5% CO_2 and also maintained at 37^oC at a pH 7.4, to ensure contractions of the heart stop.

Hearts then underwent the perfusion stage with modified KH digestion buffer (1mg/ml Gibco type II collagenase isolated from Clostridium histolyticum, 1mM $CaCl_2$). Throughout this period of perfusion with collagenase (Gibco, UK), the effluent was recirculated through the experiment to ensure a successful isolation took place (Louch et al. 2011). Once the enzyme digestion of the heart occurred, the ventricles of the heart were fragmented and incubated for 5 minutes in the orbital shaker containing digestion buffer. The supernatant was then centrifuged for 2 minutes at 600rpm and the pellet obtained was re-suspended in fresh restoration buffer (116 mM NaCl, 5.4mM KCl, 0.4mM $MgSO_4.7H_20$, 10mM glucose, 20 mM taurine, 5mM sodium pyruvate, 25 mM $NaHCO_3$, 11.75mM KH_2PO_4, 1% bovine serum albumin (BSA), 1% Pen-Strep, 5mM creatine monohydrate and 100mM $CaCl_2$, pH 7.4).

The cell viability was monitored and strictly assessed through the visualization of the cardiomyocyte cells under the inverted microscope as seen in Figure 2.8. The final concentration of calcium that needed to be added to the restoration buffer containing the cells was added gradually to a final concentration of 1.25mM. This gradual addition was to ensure calcium overload didn't occur which may damage the cells. Only cell isolations that had a cell viability of over 60% progressed to the experimental procedure.

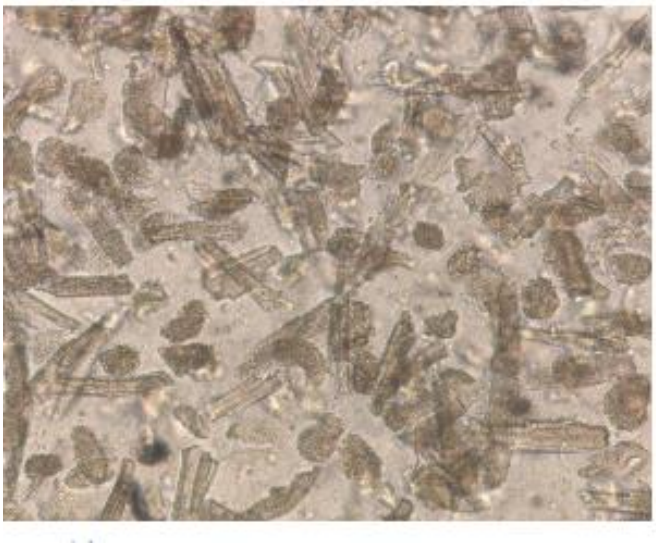


Figure 2. 8 Viable rat cardiomyocytes being observed under the inverted microscope.

2.4.1 Hypoxia Induced Isolated Rat Ventricular Cardiomyocytes

The isolated adult rat cardiomyocytes were centrifuged for 2 minute at 600rpm where the supernatant was gently removed and the pellet that contained the viable cardiac myocytes were gently redistributed in Esumi ischaemic buffer (11.95 mM KCl, 1.04mM $MgCl_2$, 1.7mM $CaCl_2$, 4mM HEPES, 10mM deoxyglucose and 20mM sodium pyruvate, pH 6.2). Cells were then transferred into a culture dish and incubated for 1 hour in a hypoxic chamber (New Brunswick Biosciences, UK) at 37^{o}C in 5% CO_2 and 0.01-1% O_2 to induce hypoxia.

Following hypoxic treatment, cells were centrifuged for 2 minutes at 600rpm where the supernatant was removed and cardiomyocytes were gently redistributed with 24ml restoration buffer at 37^{o}C. The pellet was redistributed and 1ml aliquots were pipetted into a 24 well sterile plate. Each well contained the necessary drug treatment and underwent a further 3 hours of reoxygenation. .

2.4.2 Experimental Groups in Isolated Rat Cardiomyocyte Protocol

Normoxic Control Group:

Isolated rat cardiomyocytes underwent 4 hours of reoxygenation at 37°C, 5% CO_2 and 95% O_2. Figure 2.9 represents a visual representation of the normoxic experiment.

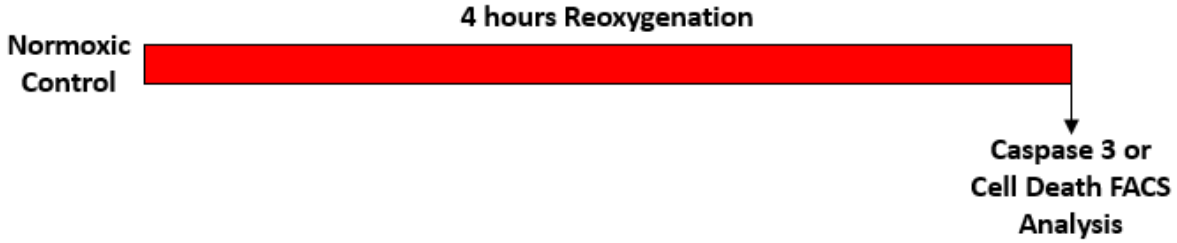


Figure 2. 9 Diagram showing the experimental protocol of the normoxic control. Isolated rat cardiomyocytes underwent 4 hours of reoxygenation.

Hypoxia/Reoxygenation (Hyp/Reox) Control Group:

Isolated rat cardiomyocytes underwent 1 hour of hypoxia at 37°C, 5% CO_2 and 0.01-1% O_2 followed by 3 hours of reoxygenation at 37°C, 5% CO_2 and 95% O_2, as seen in Figure 2.10.

Figure 2. 10 Diagram showing the experimental protocol of the Hyp/Reox control. Isolated rat cardiomyocytes underwent 1 hour of hypoxia and 3 hours of reoxygenation.

Experimental Groups at Reoxygenation:

Isolated rat cardiomyocytes underwent 1 hour of hypoxia at 37°C, 5% CO_2 and 0.01-1% O_2 followed by 3 hours of reoxygenation at 37°C, 5% CO_2 and 95% O_2. The following drugs were administered at the onset of reoxygenation to isolated rat cardiomyocytes as seen in Figure 2.11:

a) Hydrogen Peroxide was administered as a positive control.
b) A_1AR agonist 2'-MeCCPA (1nM, 10nM, 10nM and 1µM) (To determine the effects of caspase-3 activity and cell death effects of A_1AR agonist 2'-MeCCPA).
c) A_1AR agonist 2'-MeCCPA (10nM) in the presence of cell signalling cascade inhibitors – PI3K inhibitor Wortmannin (100nM); MEK1/2 inhibitor UO126 (10µM) (To determine the effects of 2'-MeCCPA (10nM) via the regulation of the PI3K-AKT and MEK1/2-ERK1/2 cell signalling pathways on caspase-3 activity and cell death).
d) A_1AR agonist 2'-MeCCPA (10nM) in the presence of selective A_1AR antagonist DPCPX (200nM) and unselective adenosine antagonist 8-SPT (1µM).

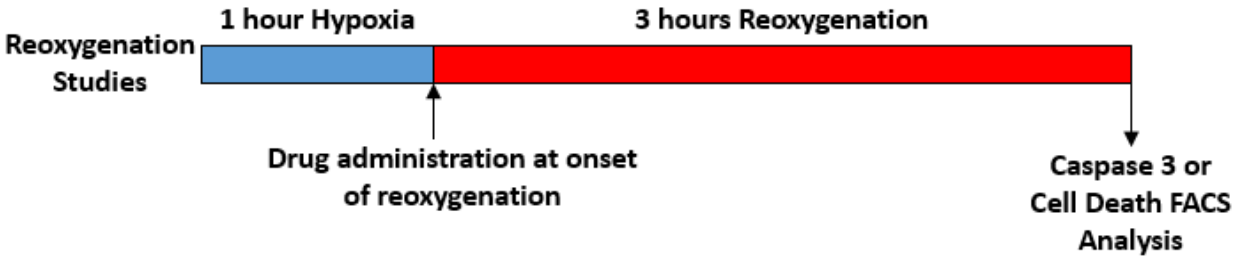


Figure 2. 11 Diagram showing the experimental protocol of the administration of all drug groups at the onset of reperfusion. Isolated rat cardiomyocytes underwent 1 hour of hypoxia and 3 hours of reoxygenation.

Experimental Groups Post-Reoxygenation:

Isolated rat cardiomyocytes underwent 1 hour of hypoxia at 37°C, 5% CO_2 and 0.01-1% O_2 followed by 3 hours of reoxygenation at 37°C, 5% CO_2 and 95% O_2. The following drugs were administered at 15 minutes or 30 minutes into the onset of reoxygenation to isolated rat cardiomyocytes, as seen in Figure 2.12:

a) Hydrogen Peroxide was administered as a positive control at 15 or 30 minutes post-reoxygenation.

b) 2'-MeCCPA (10nM) at 15 or 30 minutes post-reoxygenation.

c) 2'-MeCCPA (10nM) in the presence of PI3K inhibitor Wortmannin (100nM) or MEK1/2 inhibitor UO126 (10µM) at 15 of 30 minutes post-reoxygenation.

d) 2'-MeCCPA (10nM) in the presence of either selective A_1AR antagonist DPCPX (200nM) or unselective adenosine antagonist 8-SPT (1µM) adenosine antagonists at 15 or 30 minutes post-reoxygenation.

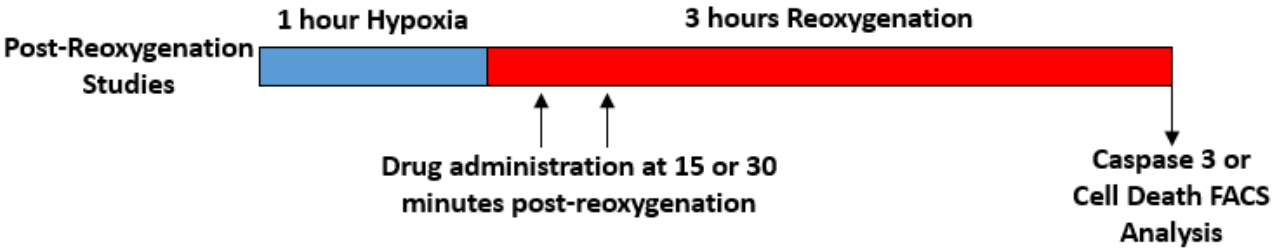


Figure 2. 12 Diagram showing the experimental protocol of the administration of all drug groups at 15 or 30 minutes post-reoxygenation. Isolated rat cardiomyocytes underwent 1 hour of hypoxia and 3 hours of reoxygenation.

2.5 Flow Cytometry Analysis

2.5.1 Experiment Protocol and Quantitative Analysis of Cleaved Caspase-3 Activity

The flow cytometry technique was used in order to evaluate caspase 3 activity following drug treatment. Once the drug treatment incubation period was completed, treated cells were transferred into labelled Eppendorf tubes and centrifuged at 1200rpm for 2 minutes. The pellet obtained was re-suspended in phosphate buffer saline (PBS) (140 mM NaCl, 5mM KCl, 1.8mM $CaCl_2$) and further fixed with 6% formaldehyde for 10 minutes at room temperature. Cardiomyocyte cells were then permeabilised by being incubated in ice cold 90% methanol for 30 minute at 4^{o}C and then stored at -20^{o}C ready for analysis.

Cells were centrifuged at 1200rpm for 2 minutes and washed twice with incubation buffer (0.5% BSA in PBS) at room temperature. The Cleaved Capase-3 (Asp175) Antibody (Alexa Fluor 488 conjugate) was prepared to a 1:100 final dilution using incubation buffer and incubated at room temperature for 1 hour in the dark. Furthermore, cells were then centrifuged at

1200rpm for 2 minutes and supernatant was discarded. The pellet was redistributed in PBS and ready for analysis.

Analysis occurred using the FL-1 channel on the BD Accuri C6 Plus Flow Cytometer. The mean fluorescence for 10 000 cell counts were investigated which indicated the cleaved-caspase 3 activity. Protocol was carried out in accordance with manufacturer's instructions (New England Biolabs, Hitchin UK).

2.5.2 Experiment Protocol and Quantitative Analysis of Cell Death using Dead Cell Apoptosis Kit with Annexin V FITC and Propidium Iodide (PI)

In preparation for flow cytometry analysis of cell death, live cardiomyocytes underwent a set of processes as recommended by the manufacturer (ThermoFischer, UK).

Cells were harvested after their incubation period and centrifuged at 1200rpm for 2 minutes. They were further washed with cold phosphate-buffered saline (PBS). The 1X annexin-binding buffer and a working solution of propidium iodide (PI) was prepared according to the manufacturer's protocol. Once cardiomyocytes had been washed, the supernatant was discarded and cells were re-suspended in 1X annexin-binding buffer. 5µl of Alexa Fluor 488 annexin V and 1 µl of pre-prepared PI working solution was added to 100 µl of cell suspension. Cells were then incubated at room temperature for 15 minutes. Next, 400 µl of 1X annexin-binding buffer was added and mixed gently and samples were then kept on ice.

Soon after, stained cells were analysed using the BD Accuri C6 Plus Flow Cytometer measuring fluorescence emission on the FL-1 channel.

2.6 Western Blotting

2.6.1 Introduction to Western Blotting

Western blotting, also known as immunoblotting, is commonly used as an analytical technique which can detect, identify and quantify fractioned proteins based on their

molecular weight, charge and conformation. The protein itself is extracted from a tissue or cell homogenate. The proteins are separated based on their size by gel electrophoresis and then further transferred onto a Polyvinyl Di Fluoride (PVDF) membrane by immunoblot transfer (Mahmood and Yang 2012; Kim 2017).

This technique is split up into four stages. Stage 1) Electrophoresis is where the separation of proteins occurs depending on their size, structure and charge; Stage 2) the transfer of the proteins onto a Polyvinyl Di Fluoride membrane (PVDF); Stage 3) incubation with the primary and secondary antibody to detect the protein of interest; Stage 4) visualisation of proteins bands of interest and analysis of their relative abundance. The molecular weight of a protein band can be compared to a reference marker. The effectiveness of this technique can be limited in a way that high levels of nonspecific background staining and limit the identification and quantification of band intensity (Olle et al. 2005).

2.6.2 Tissue Preparation and Experimental Protocols

For Western blot tissue preparation, isolated rat hearts underwent stabilisation, ischaemia and reperfusion for a specific amount of time and 2'-MeCCPA (10nM) was administered at reperfusion, 15 minutes or 30 minutes post-reperfusion in the presence and absence of PI3K inhibitor Wortmannin (100nM) or MEK1/2 inhibitor UO126 (10µM). Each protocol is described below.

At the end of specific reperfusion periods, hearts were removed from the Langendorff apparatus and the left ventricle of each heart was trimmed away using a sterile scalpel. The heart tissue was immediately wrapped in foil, labelled accordingly and snap frozen in liquid nitrogen. Then samples were stored at -80^{o}C.

2.6.3 Experimental Groups for Western Blotting

Normoxic Control Group:

Within the normoxic control group, hearts were subjected to 20 minutes of stabilisation and 135 minutes of perfusion with KHB. Heats were then taken off the Langendorff apparatus as seen in Figure 2.13.

Figure 2. 13 Protocol used to isolate heart tissue within normoxic control group for Western blot analysis.

Experimental Groups at Reperfusion Studies:

Hearts underwent 20 minutes of stabilisation, 35 minutes of ischaemia and then a further 10 minutes of reperfusion. Drug administration occurred at the onset of reperfusion and hearts were harvested for Western blot analysis after 10 minutes of reperfusion as seen in Figure 2.14. Drug groups consisted of the following:

a) Ischaemia/reperfusion control group – at the onset of reperfusion only KHB was perfused through the heart at reperfusion.
b) A_1AR agonist 2'-MeCCPA (10nM) at the onset of reperfusion.
c) A_1AR agonist 2'-MeCCPA (10nM) in the presence of cell signalling cascade inhibitor – PI3K inhibitor Wortmannin (100nM).

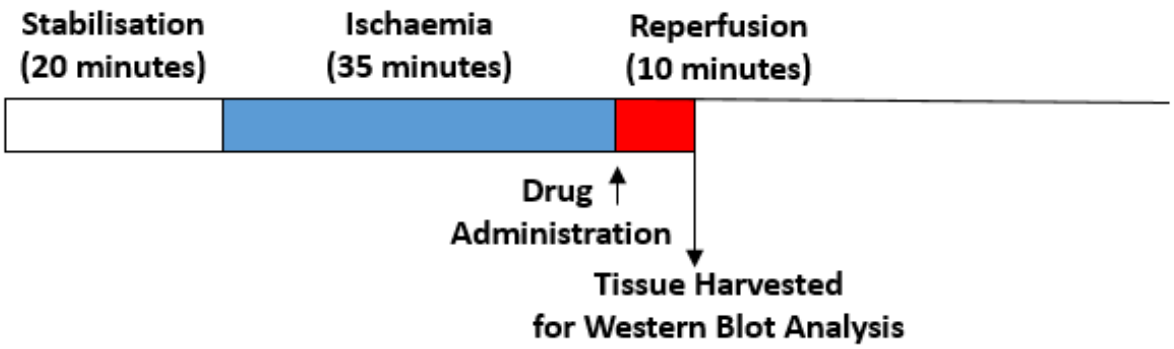


Figure 2. 14 Protocol used to isolate heart tissue with drug treatments administered at reperfusion for Western blot analysis.

Experimental Groups at 15 minutes Post-Reperfusion Studies:

Hearts underwent 20 minutes of stabilisation, 35 minutes of ischaemia and then a further 25 minutes of reperfusion or 35 minutes of reperfusion. Drug administration occurred at 15 minutes post-reperfusion and hearts were harvested either 25 minutes or 35 minutes post-reperfusion, as seen in Figure 2.15. Drug groups consisted of the following:

a) Ischaemia/reperfusion control group at 25 minutes – KHB was perfused through the hearts for 25 minutes after the onset of reperfusion.
b) Ischaemia/reperfusion control group at 35 minutes – KHB was perfused through the hearts for 35 minutes after the onset of reperfusion.
c) 2'-MeCCPA (10nM) was administered at 15 minutes post-reperfusions and hearts were harvested 25 minutes post-reperfusion.
d) 2'-MeCCPA (10nM) was administered at 15 minutes post-reperfusion and hearts were harvested 35 minutes post-reperfusion.
e) 2'-MeCCPA (10nM) was administered at 15 minutes post-reperfusion in the presence of Wortmannin (100nM). Hearts were harvested at 25 minutes post-reperfusion.
f) 2'-MeCCPA (10nM) was administered at 15 minutes post-reperfusion in the presence of Wortmannin (100nM). Hearts were harvested at 35 minutes post-reperfusion.

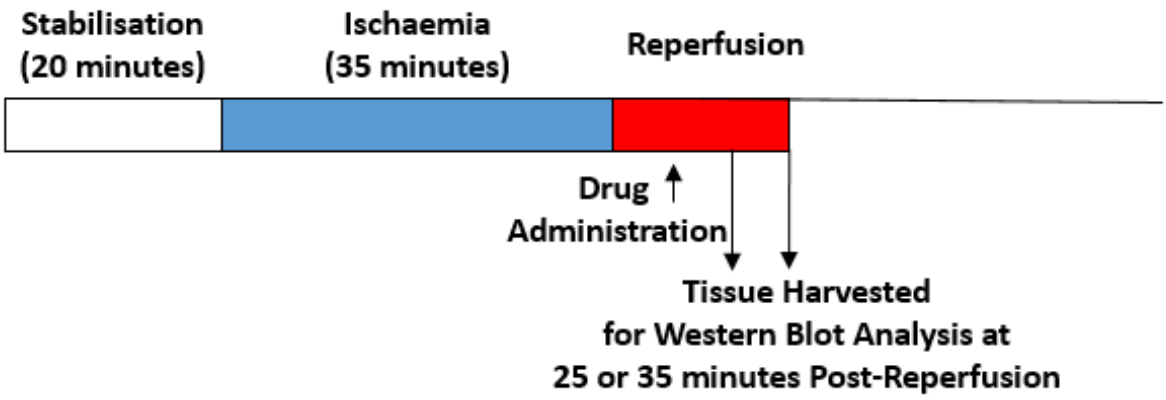


Figure 2. 15 Protocol used to isolate heart tissue with drug treatments administered at 15 minutes post-reperfusion for Western blot analysis.

Experimental Groups at 30 minutes Post-Reperfusion Studies:

Hearts underwent 20 minutes of stabilisation, 35 minutes of ischaemia and then a further 40 minutes of reperfusion or 50 minutes of reperfusion. Drug administration occurred at 30 minutes post-reperfusion and hearts were harvested either 25 minutes or 35 minutes post-reperfusion, as seen in Figure 2.16. Drug groups consisted of the following:

a) Ischaemia/reperfusion control group at 40 minutes – KHB was perfused through the hearts for 40 minutes after the onset of reperfusion.
b) Ischaemia/reperfusion control group at 50 minutes – KHB was perfused through the hearts for 50 minutes after the onset of reperfusion.
c) 2'-MeCCPA (10nM) was administered at 30 minutes post-reperfusions and hearts were harvested 40 minutes post-reperfusion.
d) 2'-MeCCPA (10nM) was administered at 30 minutes post-reperfusion and hearts were harvested 50 minutes post-reperfusion.
e) 2'-MeCCPA (10nM) was administered at 30 minutes post-reperfusion in the presence of Wortmannin (100nM). Hearts were harvested at 40 minutes post-reperfusion.
f) 2'-MeCCPA (10nM) was administered at 30 minutes post-reperfusion in the presence of Wortmannin (100nM) Hearts were harvested at 50 minutes post-reperfusion.

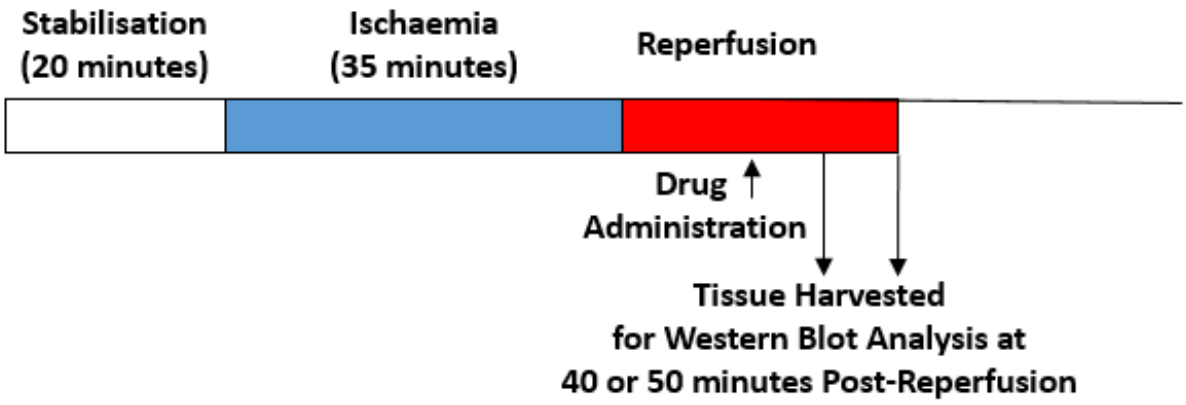


Figure 2. 16 Protocol used to isolate heart tissue with drug treatments administered at 30 minutes post-reperfusion for Western blot analysis.

2.6.4 Protein Extraction

The extraction of proteins was conducted from the stored frozen samples that were obtained previously. Approximately 60mg of the ventricular tissue sample was placed in a sterile cryogenic vial which that contained 250µl of lysis buffer (0.1M NaCl, 10mM Tris Base, 1mM EDTA (pH 8.0), 2mM Sodium phosphate, 2mM NaF, 2mM β-glycerophosphate, 1 protease cocktail tablet/100ml lysis buffer and 1 PhosSTOP tablet/10 ml lysis buffer). Following this, tissue homogenisation was carried out using IKA Ultra-Turrax Labortechnik T25 WERKE homogeniser. The homogenized samples were then centrifuged at 11 000rpm at 4^{o}C for 10 minutes. The supernatant was isolated and collected at which point it was then assessed for its protein concentration using the colorimetric Pierce™ Bicinchoninic Acid (BCA) assay kit (Thermo Fisher Scientific, UK). The sampled obtained were further diluted (2X) in sample buffer (250mM Tris-HCl pH 6.8, 10% glycerol, 0.0006% bromophenol blue, 4% SDS, 2% β-mercaptoethanol-pH 6.8) and incubated for 5 minutes at 100^{o}C before being stored at -80^{o}C. All samples were defrosted at room temperature before being used and diluted with sample buffer to obtain a protein concentration of 60µg of each sample for gel loading.

2.6.5 Protein Quantification using the BCA Protein Assay

The BCA protein assay is an accurate technique of which the colorimetric detection and measurement of total protein occurs (Olson and Markwell 2007). This technique of protein quantification is based upon the chemical reduction of Cu^{2+} to Cu^{+} by the protein in an alkaline environment using a precise and selective colorimetric exposure of cuprous cation by bicinchoninic acid. The purple colour achieved in a result from the reaction of two particles of BCA with one Cu^{1+} ion.

To fully determine the protein concentration of samples, a standard curve was created with the use of bovine serum albumin (BSA) as a reference reagent. A set of standards were prepared from 2mg/ml BSA provided within the assay. These standards were prepared as dilutions ranging from 2000µl/ml to 25µl/ml using H_2O as a diluent. Next, 25µl of each standard was added to 96 well flat-bottomed micro-titre plates and a blank standard was required also. 2µl of unknown sample protein was pipetted into distilled H_2O to obtain a dilution of 1:12.5. The overall working reagent (WR) was prepared by adding 50 parts of BCA reagent A with 1 part of reagent B (both reagent A and B were provided within the kit). 200µl of WR was pipetted into each well and mixed thoroughly using a plate shaker for around 30 seconds. The plate was incubated in the dark to eliminate any light interference in order to maximize the rate of reaction and incubated for 30 minutes at 37^{o}C. The plate was then cooled to room temperature and the absorbance was measured at 562nm using a UV/Vis plate reader. The responses received of the standards allowed for the standard curve to be calculated and plotted. Absorbance values of the unknown sampled were inserted into the standard curve created on Excel in order to determine their exact protein concentration.

2.6.6 Gel Electrophoresis

To begin the process of gel electrophoresis, 60µg of protein sample was loaded into the Biorad 4-15% Tris/Glycine precast gradient gels (Biorad, Hertfordshire, UK). The gel was then attached onto the Mini-PROTEAN 3 electrode assembly system (Biorad PowerPac 3000) and then ran at 130 volts for 90 minutes. The electrode frame was then placed within the clamping frame and the system was secured tightly and closed. The inner chamber was then lowered

into the Mini Tank which was then placed into the Biorad mini protean III system (Biorad, UK) which then went onto separate out the samples. There was approximately 125ml of 1X running buffer (glycine 14.42 g/l, SDS 1.0 g/l, TrisBase 3.0 g/l) that was added to the inner chamber of the system and approximately 200ml was added to the outer chamber of the system. The plastic combs were removed in order to expose the wells and the samples were then loaded into the appropriate wells. This was carried out by using gel loading tips (Fisher Scientific Ltd, UK). One of the wells were allocated with 2.5µl of biotinylated protein ladder (Cell Signalling Technologies, UK) which contained bands of specific molecular weight which aided in the identification of the target protein. The gel was then run using electrical leads from the Mini-PROTEAN 3 apparatus to the Power-Pac 9 (Bio-Rad, UK) at 130 volts for 90 minutes at room temperature.

2.6.7 Protein Transfer

Once the electrophoresis step was conducted and protein bands had separated, the gel was carefully removed from the glass plates and placed into the Trans-Blot Turbo transfer pack (Bio-Rad, UK), which consisted of a Polyvinyl Di Fluoride (PVDF) membrane and buffer. The transfer cassette was firmly assembled together and closed according to the manufacturer's instruction (Bio-Rad, UK) and placed into the Trans-Blot Turbo transfer system (Bio-Rad, UK). The settings on the transfer system was selected for the transfer at 25V, 1.3A for 7 minutes using two gels.

2.6.8 Immunoblotting Procedure

Once the protein transfer procedure was conducted, the PVDF membrane was incubated with freshly prepared blocking buffer (5% w/v milk powder in 1X TrisBase Tween20 (TBS-T) buffer (2.42 g/l Tris-Base, 8g/l NaCl, 1ml/l Tween20) for one hour facing upwards. The membrane was then further washed 3 times for 5 minutes in 1X TBST buffer and then incubated overnight with the primary antibody diluted to 1:1000 in antibody buffer (5% w/v BSA in 1XTBS-T) on an orbital shaker at 4^{o}C.

Primary antibodies included: phosphorylated and total-AKT$_{ser473}$, phosphorylated and total-p44/42 MAPK (ERK1/2) $_{(Thr202/Try204)}$ and GAPDH. GAPDH was used an internal loading control in all experiments to ensure that uniform loading of gel samples and uniform transfer of

proteins occurred during the immunoblotting stage. The relative variation levels of monoclonal rabbit phosphorylated-AKT_{ser473} were normalized to the total form of AKT_{ser473}. This was also conducted for the monoclonal rabbit phosphorylated-p44/42 MAPK (ERK1/2) $_{(Thr202/Try204)}$ being normalized to the total form of the rabbit monoclonal p44/42 MAPK (ERK1/2) $_{(Thr202/Try204)}$.

The membranes were then washed 3 times for 5 minutes in 1X TBST and incubated with secondary anti-rabbit antibody HRP (1:1000 dilution in antibody buffer) linked IgG and HRP linked with anti-biotin antibody (in order to visualize the biotinylated protein marker) for 1 hour on an orbital shaker. After this last stage of incubation, the membrane was washed 3 times again for 5 minutes in 1X TBST before analysis of membranes could occur using the Biorad ChemiDoc imaging system.

In order to normalise phosphorylated-AKT_{ser473} and phosphorylated-p44/42 MAPK (ERK1/2)$_{(Thr202/Try204)}$ to their total forms, membranes were placed in stripping buffer for 5 minutes and to start the re-probing process for total forms of the antibodies, membranes were incubated in blocking buffer (5% milk powder in TBST) for an hour. Then incubated overnight with the Total antibodies at a dilution of 1:1000 upon the orbital shaker at 4^{o}C. After the overnight incubation period, membranes were washed 3 times in 1X TBST and then incubated in 1:1000 dilution and incubated with secondary anti-rabbit antibody linked IgG and HRP linked with anti-biotin antibody (in order to visualize the biotinylated protein marker) Membrane blots were then visualized under the ChemiDoc imaging system (BioRad, UK).

2.6.9 Visualisation and Quantification of Band Density

Furthermore, membranes were placed on acetate film where 1ml of Super Signal West Femto Maximum Sensitivity Substrate solution (ThermoFischer Scientific, UK) was prepared by combining reagent A and B in a 1:1 dilution, and then added onto the membranes. This solution is an ultra-sensitive substrate that contains luminol which is a widely used chemiluminescent reagent. This solution covered the entire surface of the membrane within excess for 5 minutes before it was placed within the Biorad™ ChemiDoc imaging system (Bio-Rad, UK). With the aid of the Bio-Rad Quantity One software, the membrane was exposed for 10-30 seconds which allowed for the protein bands to be visualized. Band density was then

analysed by assessing the background exposure for the blot with the aid of ImageJ (N.I.H, Bethesda, USA). The target protein was then accurately identified.

Data was presented as a mean±SEM. Statistical differences were calculated using the IBM SPSS programme where one-way ANOVA with LSD post-hoc test was conducted. Significance was considered at $p<0.05$.

2.7 Statistical Analysis of All Techniques Used

All data that was presented in this project is expressed at the mean ± standard error of the mean (SEM). IBM Statistical Package for Social Sciences (SPSS®v21) software was used to statistically analyse the data. The statistical tests currently used to analyse infarct sizes, band densities and cell population data was by one-way ANOVA accompanied by Fishers Protected Least Significant Difference (LSD) test for multiple comparisons. To assess the difference in the data sets, a p-value of $p<0.05$ was used to consider statistical significance.

All data was presented in graphs made on Microsoft Excel.

Chapter 3: Profiling of A_1 adenosine receptor agonist, 2'-MeCCPA within the ischaemia-reperfusion injury model in isolated rat heart and rat cardiomyocytes

3.1 Introduction

3.1.1 Adenosine and Adenosine Receptors

Adenosine is an endogenous nucleoside modulator and it is released from almost all cells. It is generated by the dephosphorylation of ATP into AMP which is further broken down into adenosine. Adenosine has the ability to be released as a response to an injurious effect that has occurred for example in response to an ischaemic insult (Gessi et al. 2011; Boreo et al. 2016).

There are currently four different adenosine receptors that belong to a superfamily of G-protein coupled receptors (GPCR). These receptors can be categorised as A_1, A_{2A}, A_{2B} and A_3 receptors and each of them cause's stimulatory and inhibitory actions on adenylate cyclase. Each receptor is selective to their respective agonists and antagonists (Boreo et al. 2016; Fredholm 2014; Covinhes et al. 2020; Gaudry et al. 2020).

The A_1 adenosine receptor itself is coupled to the pertussis toxin sensitive G_i and G_o proteins that go on to inhibit adenylate cyclase activity which can further inhibit cAMP activation as therefore activate the opening to K^+ ion channels and the inhibition of Ca^{2+} release (Germack and Dickenson 2004). The A_3 adenosine receptor is also coupled to G_i and G_o as well as G_q proteins which in turn makes their functioning very similar to the A_1 adenosine receptor. The A_1 adenosine receptor has been well researched to decrease heart rate in many studies (Albrecht-Kupper, Leineweber and Nell 2012; Hoffman et al. 1997).

3.1.2 A_1 Adenosine Mediated Cardioprotection

Previous research has been conducted to express how pharmacological pre-conditioning with A_1AR agonist can mediate cardioprotection, for example Hill et al. (1998) were able to mediate cardioprotection within the rabbit heart model with the administration of adenosine. This study was able to identify and support studies in showing that selective A_1 adenosine

receptor agonists were able to pharmacologically mimic ischemic pre-conditioning to ensure cardioprotection within the rabbit heart through the A_1 adenosine receptor (Hill et al. 1998). To further support the essence of cardioprotection in animal models, Hochhauser et al. (2007) were able to mediate cardioprotection with pre-ischaemic pharmacological preconditioning (PC) in the perfused rat isolated heart model with the use of A_1 adenosine receptor agonist, 2-Chloro-N^6-cyclopentyladenosine (CCPA), the protection that occurred partially related to the increased phosphorylation of p38 MAPK (Hochhauser et al. 2007).

Post-conditioning is another phenomenon in which the A_1 adenosine receptor, when activated, has the ability to induce infarct limiting cardioprotection (Xi et al. 2008). Post-conditioning is a concept of briefs periods of coronary occlusion performed at the beginning of reperfusion which have shown to be successful (Pagliaro and Penna 2011); however confounding research has also been established by Kin et al. (2005) who have suggested that post-conditioning protection to the myocardium is not via the A_1AR subtypes but more so via the A_{2A} and A_3AR subtypes.

The phenomenon of post-conditioning differs from post-reperfusion activation in a way that in post-reperfusion activation, rat hearts are undergoing 35 minutes of simulated ischaemia and then 120 minutes of reperfusion in which A_1 adenosine receptor agonist 2'-MeCCPA is administered at the onset of reperfusion and 15 or 30 minutes into reperfusion. Whereas post-conditioning would include brief periods of ischaemia to be simulated after the 35 minutes of simulated ischaemia with A_1 agonist 2'-MeCCPA being administered throughout reperfusion to assess the effects of it. This makes the current study different from post-conditioning. This idea of post-reperfusion activation can also be supported by Von Lubitz and colleagues (2001) who found that A_3 adenosine receptor agonist IB-MECA, when administered 20 minutes post-reperfusion to the brain was able to significant increase protection after an ischaemic injury. Further research conducted by Hussain (2009) showed that the post-reperfusion activation of A_3 adenosine receptors using Cl-IB-MECA successfully attenuated infarct size to risk ratio (%) as well as cell death and caspase-3 activity. With such extensive research conducted into the post-reperfusion A_3AR activation as well as the similarities between the A_3 and the A_1 adenosine receptors, this became a basis for the current study conducted to explore the post-reperfusion activation of the A_1 adenosine receptor.

Taking into consideration the previous research conducted into the A_3AR itself, this current study was conducted to look into the cardioprotective effects of activation of the A_1ARs at reperfusion and post-reperfusion rather than through the pre-conditioning and post-conditioning phenomenon's. This idea of post-reperfusion activation of A_1 adenosine receptors is an area of research that has not currently been fully explored.

3.1.3 Aims and Objectives

1. To determine the cardioprotective effects of A_1 adenosine receptor (A_1AR) agonist, 2'-MeCCPA (0.1nM - 1μM) in a dose-dependent manner on infarct size to risk ratio (%), cell death (apoptosis and necrosis) and caspase-3 activity when administered at the onset of reperfusion/reoxygenation.
2. To determine the post-reperfusion/reoxygenation activation effects of A_1 adenosine receptors using A_1AR agonist 2'-MeCCPA (10nM) when administered at the onset of reperfusion compared to the administration 15 minutes and 30 minutes post-reperfusion/reoxygenation; and the effects upon infarct size to risk ratio (%), cell death (apoptosis and necrosis) and cleaved caspase-3 activity.

3.2 Methods

3.2.2 Chemicals

2'-MeCCPA was purchased from Tocris Cookson (Bristol, UK). Working concentrations of the drug used within this chapter were freshly prepared each day as described in Chapter 2, Section 2.2.

3.2.3 Animals

Adult male Sprague-Dawley rats (350 ± 50g) were sacrificed via cervical dislocation outlined in the Schedule 1 Home Office Procedure using the process of thoracotomy, previously described in Chapter 2, Section 2.1.

3.2.4 Langendorff Protocol – Isolated Perfused Rat Heart Preparation

Briefly, for studies using the Langendorff model of ischaemia-reperfusion (details in Chapter 2, Section 2.3.5), the experiments were carried out for 175 minutes in total. Hearts were allowed a 20 minutes stabilisation period, followed by 35 minutes of simulated regional ischaemia and 120 minutes of reperfusion. Hearts were random allocated to the following control and treatment groups:

a) Normoxic control – Rat hearts perfused with KH buffer for 175 minutes (no ischaemia was induced).
b) Ischaemia-Reperfusion (IR) Control – Rats hearts were perfused with KH buffer for 20 minutes followed by 35 minutes of simulation regional ischaemia and then 120 minutes of reperfusion.
c) Concentration response – Rats were perfused with KH buffer for 20 minutes, 35 minutes of simulated regional ischaemia and 120 minutes of reperfusion. Throughout the reperfusion stage, hearts were allocated with various concentrations of A_1AR agonist, 2'-MeCCPA (0.1nM, 1nM, 10nM, 100nM and 1µM).
d) Time-point response – Rats were perfused with KH buffer for 20 minutes, 35 minutes of simulated regional ischaemia and 120 minutes of reperfusion were 2'-MeCCPA (10nM) was administered at the onset of reperfusion or 15 minutes post-reperfusion or 30 minutes post-reperfusion

Throughout the course of the experiments, the stability of the hearts were monitored through the heart rate (HR), left ventricular developed pressure (LVDP) and coronary flow (CF) parameters (as described in Chapter 2, Section 2.3.2).

At the end of the reperfusion period, the infarct size to risk ratio was assessed with the use of Evans blue triphenyltetrazolium chloride (TTC) staining procedures as described in Chapter 2, Section 2.3.6.

3.2.5 Isolation of Adult Rat Ventricular Cardiomyocytes

Adult rat ventricular cardiomyocytes were isolated by enzymatic digestion as previously described in Chapter 2, Section 2.4. All details of the heart digestion protocol and conditions in order to conduct a successful isolation of primary adult rat cardiomyocytes are described in Chapter 2, Section 2.4.

3.2.6 Induction of Hypoxia and Reoxygenation Conditions in Adult Rat Cardiomyocytes

As described in Chapter 2, Section 2.4.1, freshly isolated ventricular myocytes were incubated in Esumi hypoxic buffer for 1 hour in a hypoxic chamber at 37^{o}C, in 5% CO_2 and 0.01-1% O_2 to induce hypoxia.

3.2.7 Experimental Drug Treatment Protocol in Adult Rat Ventricular Cardiomyocytes

Isolated rat ventricular cardiomyocytes were exposed to differing control and drug treatments. All conditions are detailed below:

a) Normoxic control – Isolated myocytes were exposed to normoxic conditions for a total of 4 hours at 37^{o}C, 5% CO_2 and 95% O_2.
b) Hypoxia-Reoxygenation control – Isolated myocytes were exposed to 1 hour of hypoxia conditions followed by the onset of reoxygenation for 3 hours.
c) Concentration response – Isolated myocytes were exposed to 1 hour of hypoxia conditions followed by treatment with 2'-MeCCPA (1nM, 10nM, 100nM and 1µM) at the onset of reoxygenation for 3 hours at 37^{o}C, 5% CO_2 and 95% O_2.
d) Time-point response – Isolated myocytes were exposed to 1 hour of hypoxia conditions followed by treatment with 2'-MeCCPA (10nM) at either; the onset of reoxygenation, 15 minutes post-reoxygenation or 30 minutes post-reoxygenation for 3 hours at 37^{o}C, 5% CO_2 and 95% O_2.

3.2.8 Quantitative Analysis of Cell Death using Dead Cell Apoptosis Kit with Annexin V FITC with Propidium Iodide (PI) in Adult Rat Cardiomyocytes

Followed by the drug treatment protocols as described above, isolated rat cardiomyocytes were assess for their levels of apoptosis and necrosis using the Dead Cell Apoptosis Kit with Annexin V FITC and PI was purchased from ThermoFisher (UK) (previously detailed in Chapter 2, Section 2.5.2). Data was normalised against the cell only control and the values obtained were calculated as a relative change in apoptosis and necrosis activity of the mean absorbance of the control group.

3.2.9 Analysis of Cleaved Caspase-3 Activity in Adult Rat Cardiomyocytes

Following the drug treatment protocol as described above, the rat cardiomyocytes were probed with cleaved caspase-3 antibody as described in Chapter 2, Section 2.5.1. Data obtained was normalised to the cell only control by subtracting the mean fluorescence background recorded in untreated samples. Data was presented as a relative change in fluorescence activity.

3.2.10 Data analysis

All data that was presented in this project is expressed at the mean ± standard error of the mean (SEM). IBM Statistical Package for Social Sciences (SPSS®) software was used to statistically analyse the data. The statistical tests currently used to analyse infarct sizes, band densities and cell population data was by one-way ANOVA accompanied by Fishers Protected Least Significant Difference (LSD) test for multiple comparisons. To assess the difference in the data sets, a p-value of $p<0.05$ was used to consider statistical significance.

Microsoft Excel was also used to and present all data graphically.

3.3 Results

3.3.2 Profiling of various concentrations of 2'-MeCCPA (0.1nM - 1µM) in myocardial ischaemia reperfusion injury within isolated perfused rat hearts.

3.3.2.1 The effects of 2'-MeCCPA (0.1nM-1µM) when administered at the onset of reperfusion on haemodynamic parameters (left ventricular developed pressure, heart rate and coronary flow).

Haemodynamic parameters such as the left ventricular developed pressure, heart rate and coronary flow were consistently monitored in Langendorff studies. Hearts were subjected to 20 minutes of stabilisation, then 35 minutes of ischaemia followed by 120 minutes of reperfusion with 2'-MeCCPA (0.1nM, 1nM, 10nM, 100nM and 1µM) administered throughout the reperfusion period.

A general decrease in left ventricular developed pressure (LVDP) was observed after 5 minutes of regional ischaemia in the ischaemia-reperfusion (IR) control and 2'-MeCCPA (0.1nM-1µM) groups. It was found that there was a significant increase in LVDP within the normoxic group compared to all other treatment groups ($P>0.05$) (Figure 3.1). No significant difference between the groups at any of the time-points throughout reperfusion was detected ($p>0.05$ Figure 3.1).

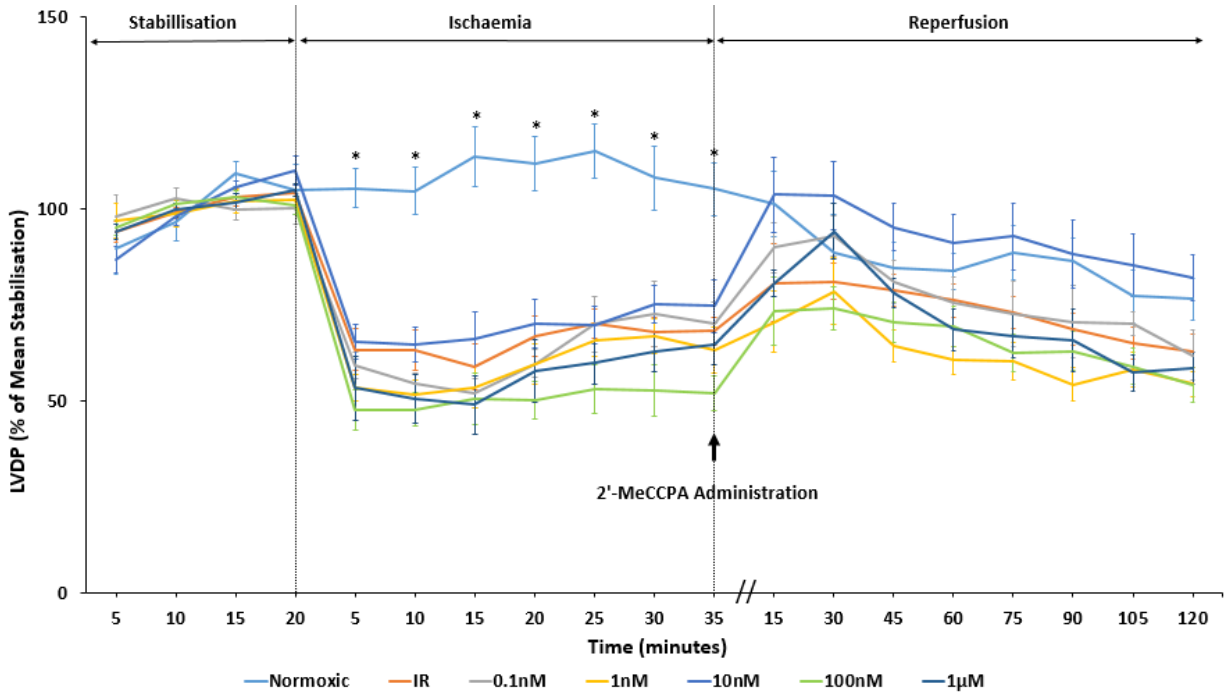


Figure 3. 1 Assessing the effects of 2'-MeCCPA (0.1nM-1µM) on left ventricular developed pressure on isolated rat hearts that were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (0.1nM-1µM) was administered throughout reperfusion. Data presented as Mean±SEM. n=6-8. * $p<0.05$ normoxia vs. all treatment group (ischaemia).

A significant decrease in heart rate was detected when hearts were administered with 2'-MeCCPA (100nM) at each time point within reperfusion compared to the normoxia group ($p<0.05$) (Figure 3.2).

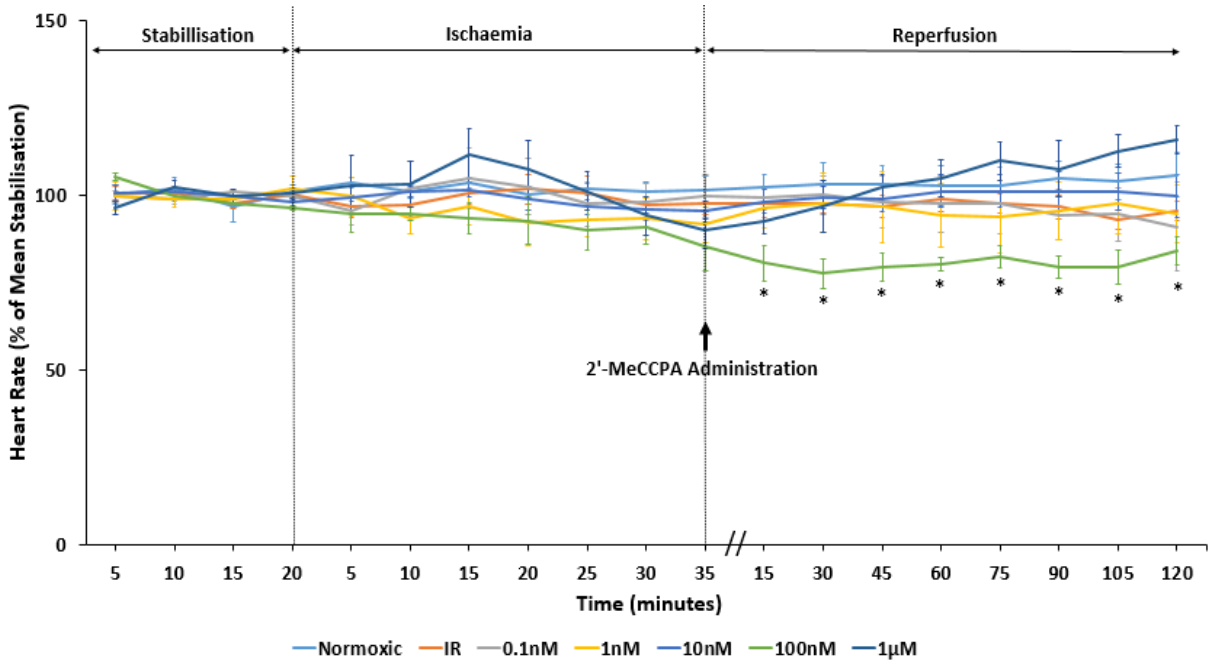


Figure 3. 2 Assessing the effects of 2'-MeCCPA (0.1nM-1µM) on heart rate within isolated rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes of reperfusion. 2'-MeCCPA groups (0.1nM-1µM) was administered at the onset and throughout reperfusion. Data was presented as Mean±SEM. n=6-8. * $p<0.05$ normoxia vs. 100nM.

Following left coronary artery occlusion, the coronary flow decrease vastly after 5 minutes of ischaemia within all groups. When 2'-MeCCPA (0.1nM-1µM) was administered throughout reperfusion, no significant effect upon coronary flow was detected compared with the time matched control ($p>0.05$, Figure 3.3). Interestingly, 2'-MeCCPA (0.1nM - 1µM) treated hearts did all increase the coronary flow in comparison to the time matched IR control heart. No statistical significance was observed.

Throughout the period of ischaemia, all treatment groups significantly decreased the coronary flow in comparison to the normoxic control ($p<0.05$) (Figure 3.3).

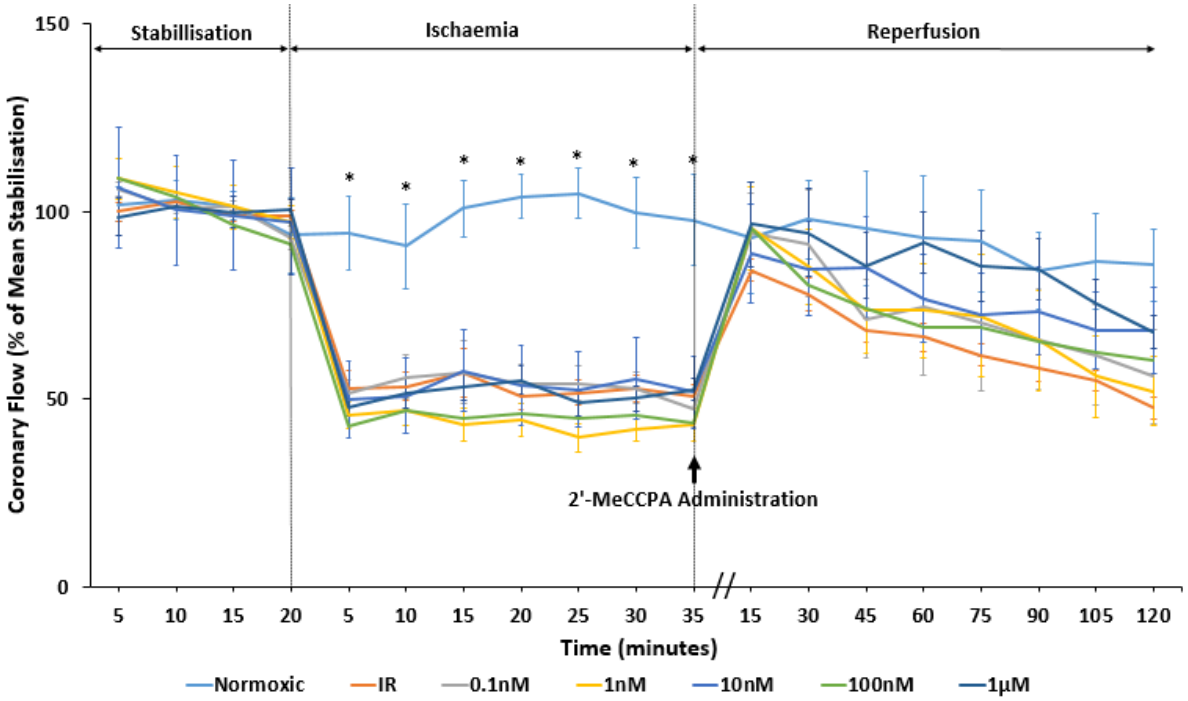


Figure 3. 3 Assessing the effects of 2'-MeCCPA (0.1nM-1µM) on coronary flow in isolated rat hearts that were subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (0.1nM - 1µM) was administered at the onset of reperfusion. Data was presented as Mean±SEM. n=6-8. * $p<0.05$ normoxia vs. all treatment group (ischaemia).

3.3.2.2 Profiling effects of 2'-MeCCPA (0.1nM-1µM) on infarct size to risk ratio within isolated hearts subjected to ischaemia reperfusion injury.

All hearts were subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion in the presence and absence of the varying concentrations of 2'-MeCCPA (0.1nM-1µM). These concentrations were administered throughout reperfusion and followed by TTC staining to determine the infarct size to risk ratio (%).

The area at risk (ischaemic area) was quantified for each heart to ensure the values do not vary significantly between the groups. This allows for better assessment and quantification of the % of infarcted tissues as well as for more reliable quantification of drug treatment. Quantifying infarct size as a percentage of area of risk between the groups corrects for variability in heart sizes and induced ischaemia between the different hearts and also the groups.

There was a significant increase in infarct size to risk ratio (%) when the ischaemia-reperfusion control (IR) was compared to the Normoxic control (55 ± 6% IR control vs. 7 ± 2% Normoxic control, p<0.001) (Figure 3.4).

The administration of various concentrations of 2'-MeCCPA (0.1nM-1µM) to the myocardium had different effects upon the infarct size to risk ratio (%). From Figure 3.4 the administration of 2'-MeCCPA (10nM) in particular throughout reperfusion showed the most significant decrease in infarct size to risk ratio (%) when compared to the IR control (28 ± 4% vs. 55 ± 6%, p<0.001), this concentration was then taken on for further study throughout this chapter. A significant decrease in infarct size to risk ratio, when compared to the IR control, was also observed at the concentrations of 1nM (42 ± 8% vs. 55 ± 6%, p<0.01), as well as at the concentration of 100nM (32 ± 8% vs. 55 ± 6%, p<0.01) and also at 1µM (33 ± 6% vs. 55 ± 6%, p<0.01).

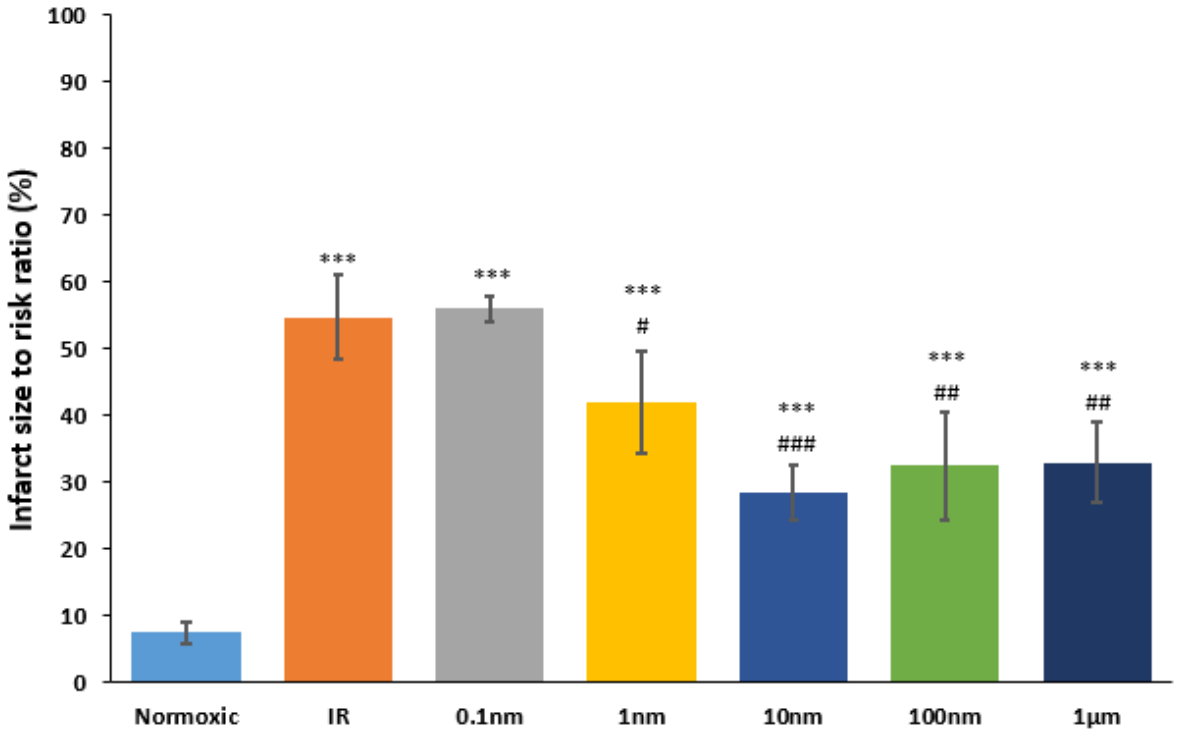


Figure 3. 4 Infarct size to risk ratio (%) within isolated perfused hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence and absence of either 2'-MeCCPA (0.1nM, 1nM, 10nM, 100nM and 1µM) throughout the reperfusion period. Data presented as Mean±SEM. n=6-8. *** p<0.001 vs. normoxic, ## p<0.01 vs. IR, ## p<0.01 vs. IR and # p<0.05 vs. IR.

3.3.2.3 Profiling effects of 2'-MeCCPA (0.1nM-1μM) on apoptosis and necrosis when administered at reoxygenation within adult rat cardiomyocytes subjected to hypoxia-reoxygenation

Isolated rat cardiomyocytes were subjected to different protocols as described in Chapter 2. Isolated cardiomyocytes were then either allowed to reoxygenate for a total of 4 hours (normoxic control group) or exposed to 1 hour of hypoxia followed by 3 hours of reoxygenation (Hyp/Reox control group) in the presence and absence of A_1AR agonist, 2'-MeCCPA (1nM-1μM) at the onset of reoxygenation within this section. After the cells underwent reoxygenation they were assessed on the flow cytometer to further determine the percentage levels of live, apoptotic and necrotic cells out of 10000 cells.

Isolated adult rat myocytes were subjected to 1 hour of hypoxia followed by 3 hours of reoxygenation which then resulted in a 261% increase within the number of apoptotic cells compared to the normoxic group (34±6% Hyp/Reox vs. 13±5% Normoxia, $p<0.001$) (Figure 3.5). The isolated rat cardiomyocytes subjected to 1 hour of hypoxia followed by 3 hours of reoxygenation also resulted in a 280% increase within the number of necrotic cells compared to the normoxia group (28±7% Hyp/Reox vs. 11±3% Normoxia, $p<0.001$) (Figure 3.5).

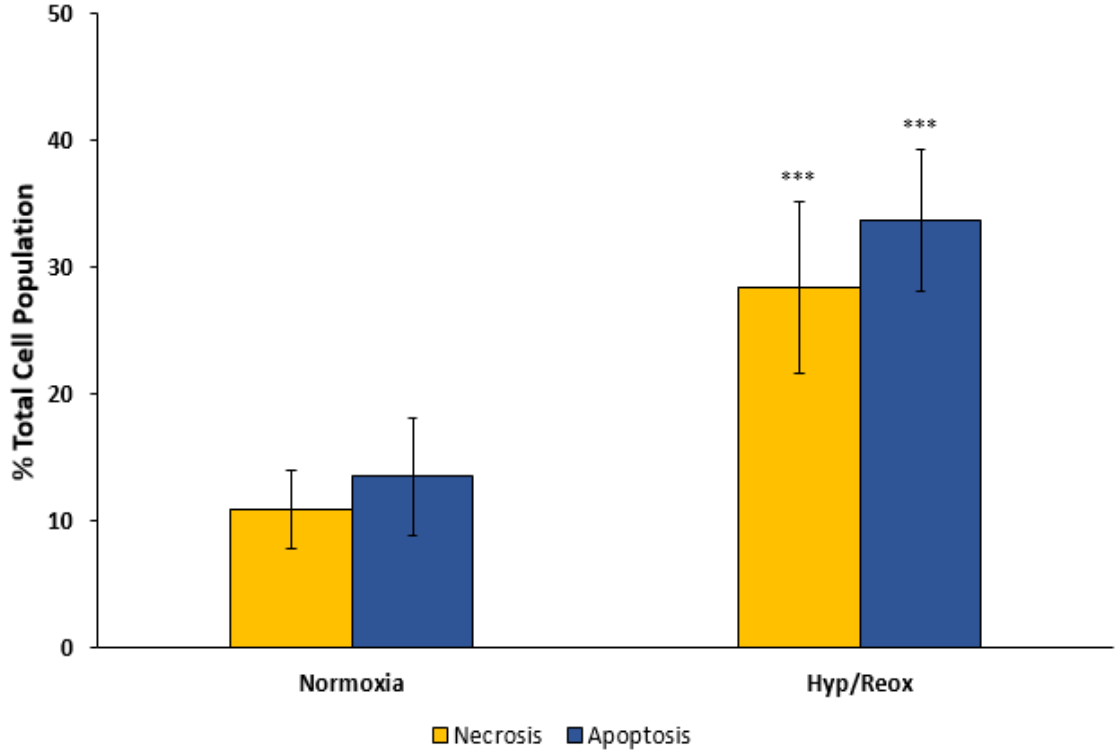


Figure 3. 5 The assessment of apoptosis and necrosis within isolated adult rat cardiomyocytes subjected to 4 hours of oxygenation (Normoxia) or 1 hour of hypoxia and 3 hours of reoxygenation (Hyp/Reox). Results are shown as Mean±SEM and are expressed as a percentage of 10000 cells counted from 6 individual experiments. *** $p<0.001$ Hyp/Reox vs. Normoxia.

Studies were conducted to ascertain the role of A_1ARs in limiting the deleterious consequences of reoxygenation injury within adult rat cardiomyocytes that were subjected to 1 hour of hypoxia and 3 hours of reoxygenation where A_1AR agonist 2'-MeCCPA (1nM, 10nM, 100nM and 1μM) was administered at the onset and throughout the reoxygenation period. Differing concentrations of 2'-MeCCPA (1nM, 10nM, 100nM and 1μM) was administered at reperfusion in order to determine the cytoprotective effects of 2'-MeCCPA and if cytoprotective effects increased as the concentrations of 2'-MeCCPA increased.

The administration of A_1 adenosine receptor agonist 2'-MeCCPA (1nM) throughout reoxygenation significantly decreased the percentage of apoptotic cardiomyocytes when compared to the Hyp/Reox group (18±2% 2'-MeCCPA (1nM) vs. 34±6% Hyp/Reox, $p<0.001$) (Figure 3.6). The administration of 2'-MeCCPA (2nM) throughout reoxygenation also significantly decreased the percentage of necrotic cardiomyocytes when compared to the Hyp/Reox control group (15±3% 2'-MeCCPA (1nM) vs. 28±7% Hyp/Reox, $p<0.001$) (Figure 3.6).

At higher concentrations of 2'-MeCCPA (10nM, 100nM and 1µM), significant protection from hypoxia-reoxygenation injury was observed through an anti-apoptotic and anti-necrotic manner. The administration of 2'-MeCCPA (10nM) throughout reoxygenation significantly decreased the percentage of apoptotic cardiomyocytes compared to Hyp/Reox (17±4% 2'-MeCCPA (10nM) vs. 34±6% Hyp/Reox, $p<0.001$). When 2'-MeCCPA (10nM) was administered throughout reoxygenation, it also significantly decreased necrotic cardiomyocytes (13±2% 2'-MeCCPA (10nM) vs. 28±7% Hyp/Reox, $p<0.001$) (Figure 3.6). Administration of 2'-MeCCPA (100nM) throughout the duration of reoxygenation significantly decreased the number of apoptotic cardiomyocytes compared to the Hyp/Reox group (19±2% 2'-MeCCPA (100nM) vs. 34±6% Hyp/Reox, $p<0.001$). The administration of 2'-MeCCPA (100nM) also significantly decreased the number of necrotic cells when compared to Hyp/Reox group (15±2% 2'-MeCCPA (100nM) vs. 28±7% Hyp/Reox, $p<0.001$). Lastly, the administration of 2'-MeCCPA (1µM) significantly decreased the number of apoptotic myocytes in comparison with the Hyp/Reox group (17±7% 2'-MeCCPA (1µM) vs. 34±6% Hyp/Reox, $p<0.001$). Furthermore, the administration of 2'-MeCCPA (1µM) also significantly decreased the number of necrotic cells compared to the Hyp/Reox group (16±2% 2'-MeCCPA (1µM) vs. 28±7% Hyp/Reox, $p<0.001$) (Figure 3.6).

Due to the number of apoptotic and necrotic cardiomyocytes being the least within the 2'-MeCCPA (10nM) group, the concentration of 10nM of 2'-MeCCPA was used in all further experiments unless otherwise stated.

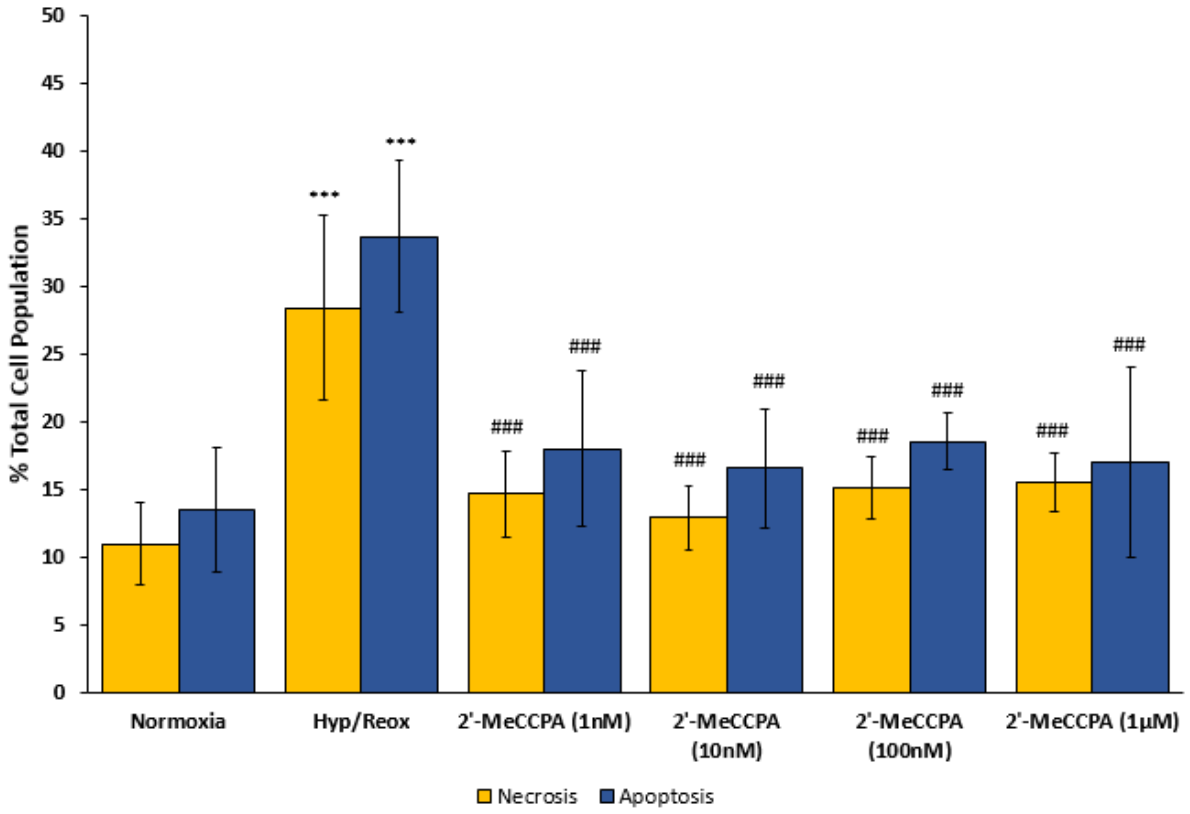


Figure 3. 6 The assessment of apoptosis and necrosis within isolated rat cardiomyocytes subjected to 1 hour hypoxia and 4 hours of reoxygenation. The A_1AR agonist 2'-MeCCPA (1nM, 10nM, 100nM and 1µM) was added at the onset of reoxygenation. Results are shown as Mean±SEM and are also expressed as a percentage of 10000 cells counted from 6 individual experiments. *** p<0.001 vs Normoxia. ### p<0.001 vs. Hyp/Reox.

3.3.2.4 Profiling effects of 2'-MeCCPA (1nM-1µM) when administered at reoxygenation on cleaved caspase-3 activity in isolated rat cardiomyocytes when subjected to 1 hour of hypoxia and 3 hours of reoxygenation

Isolated adult rat cardiomyocytes were subjected to 1 hour of hypoxia and a further 3 hours of reoxygenation resulted in a 313% increase in cleaved-caspase 3 activity when compared with the normoxic group which consisted of a 4 hours of oxygenation (313±35% Hyp/Reox vs. 100±26% Normoxia, p<0.001) (Figure 3.7). Administration of 2'-MeCCPA (1nM) throughout reoxygenation significantly decreased cleaved-caspase 3 activity when compared to the Hyp/Reox control (224±38% 2'-MeCCPA (1nM) vs. 313±35% Hyp/Reox, p<0.01) (Figure 3.7). When 2'-MeCCPA (10nM) was administered throughout reoxygenation, a significant decrease was also seen in cleaved-caspase 3 activity compared to the Hyp/Reox control group

(181±44% 2'-MeCCPA (10nM) vs. 313±35% Hyp/Reox, p<0.001) (Figure 3.7). Another significant decrease was detected when 2'-MeCCPA (100nM) was administered at the onset of reoxygenation in comparison to Hyp/Reox group (237±33% 2'-MeCCPA (100nM) vs. 313±35% Hyp/Reox, p<0.05) (Figure 3.7). When 2'-MeCCPA (1μM) was administered throughout reoxygenation, there was a decrease in cleaved-caspase 3 activity when compared to Hyp/Reox group however this was not a significant change (277±47% 2'-MeCCPA (1μM) vs. 313±35% Hyp/Reox, p>0.05) (Figure 3.7).

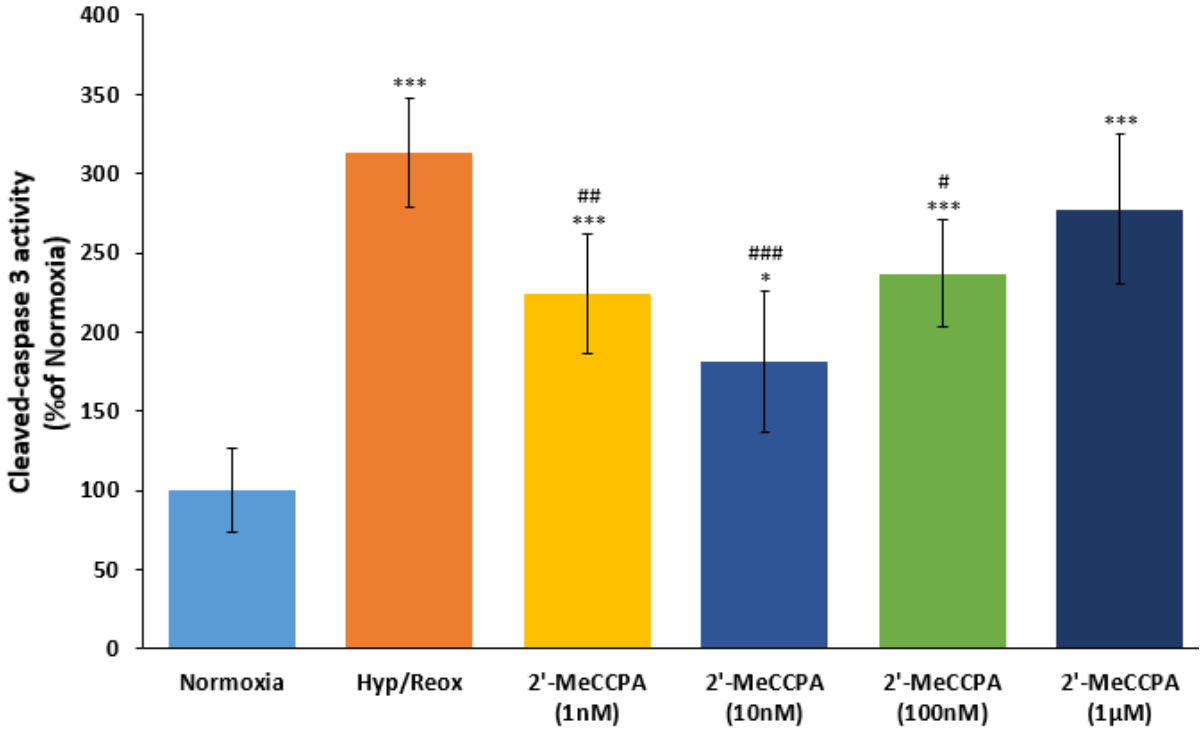


Figure 3. 7 Cleaved-caspase 3 activity within isolated adult rat cardiomyocytes subjected to 4 hours of oxygenation (Normoxia) or 1 hours of hypoxia followed by 3 hours of reoxygenation (Hyp/Reox). The A_1 adenosine receptor agonist 2'-MeCCPA (1nM, 10nM, 100nM and 1μM) was administered at the onset of reoxygenation. *** p<0.001 vs. Normoxia. * p<0.05 vs. Normoxia. ## p<0.01 vs. Hyp/Reox. ### p<0.001 vs. Hyp/Reox. # p<0.05 vs. Hyp/Reox. Data is expressed as Mean±SEM of 6 experiments.

3.3.3 The administration of 2'-MeCCPA (10nM) at various time-points within reperfusion in myocardial ischaemia reperfusion injury.

3.3.3.1 The effects of 2'-MeCCPA (10nM) at various time-points within reperfusion on haemodynamic parameters (left ventricular developed pressure, heart rate and coronary flow).

Isolated hearts were subjected to 20 minutes of stabilisation, then 35 minutes of ischaemia followed by 120 minutes of reperfusion where 2'-MeCCPA (10nM) was administered at the onset of reperfusion compared to 2'-MeCCPA (10nM) being administered at 15 minutes into the onset of reperfusion and 30 minutes into the onset of reperfusion.

A decrease in LVDP was detected in the following 5 minutes of regional ischaemia within non-treated IR control hearts as well as 2'-MeCCPA (10nM) groups administered at different time-points. Once reperfusion commenced, an increase in LVDP was detected with a gradual decline within all groups compared to the non-treated IR control group (Figure 3.8).

Throughout the ischaemic period, all treatment groups had a significantly decreased LVDP in comparison to the normoxic control ($p<0.05$) (Figure 3.8).

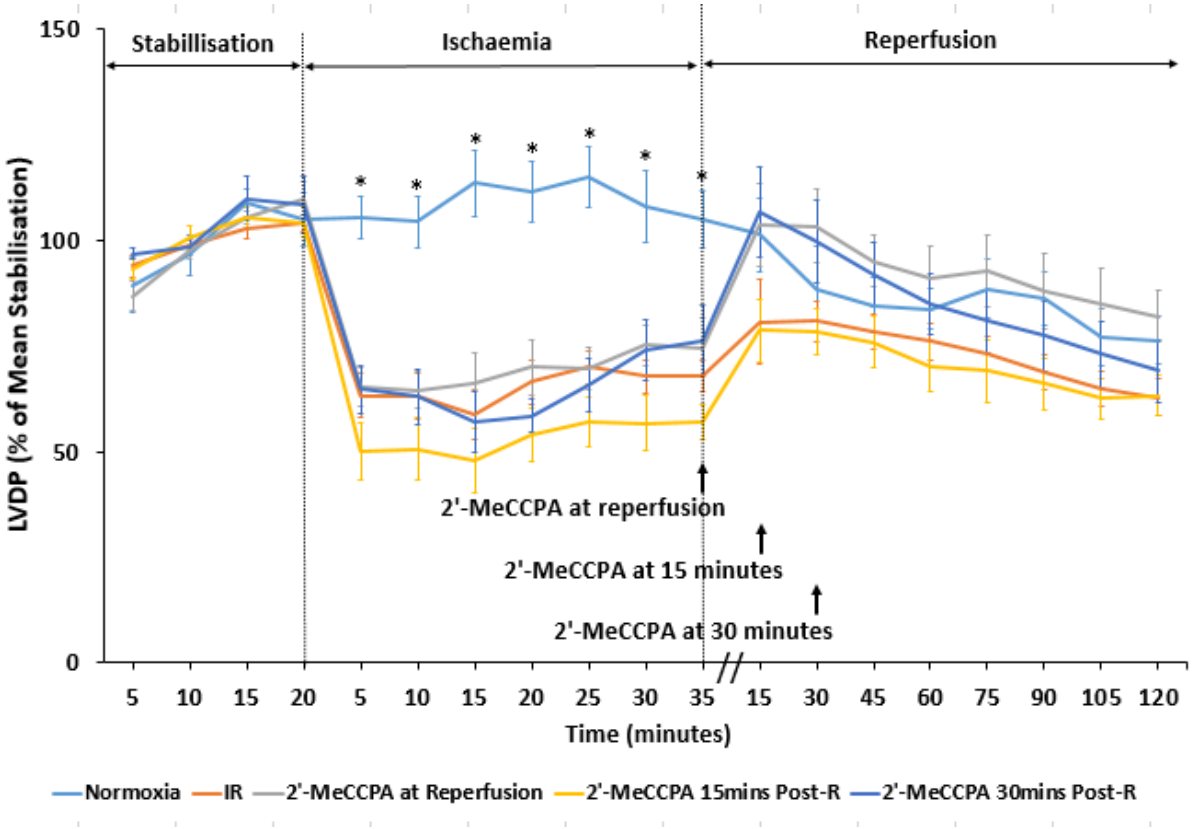


Figure 3. 8 The effect of 2'-MeCCPA (10nM) on left ventricular developed pressure on isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at the onset of reperfusion, 15 minutes post-reperfusion and 30 minutes post-reperfusion. Data was presented as Mean±SEM. n=6-8. * $p<0.05$ vs. Normoxia.

Minimum fluctuation in heart rate was observed throughout the Langendorff protocol with no statistical significance shown from 2'-MeCCPA (10nM) groups at any time-points when compared to time matched controls (p>0.05) (Figure 3.9)

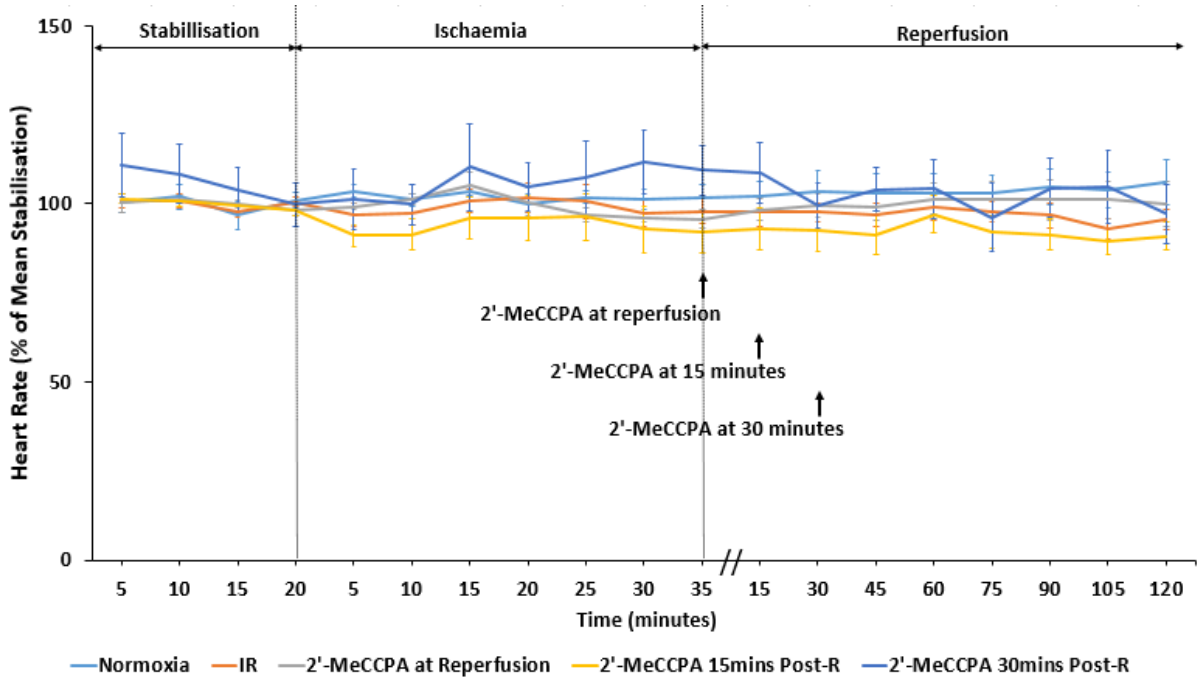


Figure. 9 The effects of 2'-MeCCPA (10nM) on heart rate on isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at the onset of reperfusion, at 15 minutes post-reperfusion and at 30 minutes post-reperfusion. Data was presented as Mean±SEM. n=6-8.

When 2'-MeCCPA (10nM) was administered at the onset of reperfusion, 15 minutes post-reperfusion and 30 minutes post-reperfusion there was a steady decline in coronary flow detected when compared to the IR control. No significant differences were detected between 2'-MeCCPA (10nM) groups compared to the IR control at any time-points within the reperfusion period (p>0.05) (Figure 3.10).

Throughout the ischaemic period, all treatment groups had a significantly decreased the coronary flow in comparison to the normoxic control (p<0.05) (Figure 3.10).

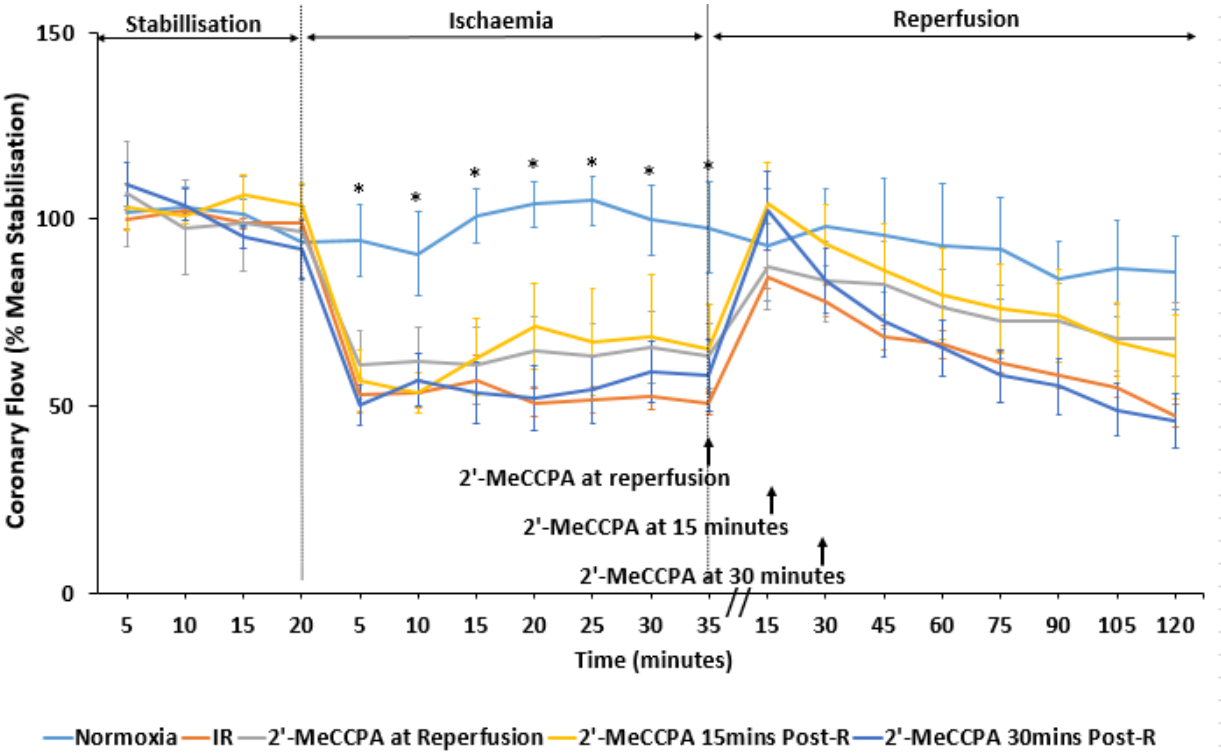


Figure 3. 10 The effects of 2'-MeCCPA (10nM) on heart rate on isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at the onset of reperfusion, at 15 minutes post-reperfusion and at 30 minutes post-reperfusion. Data was presented as Mean±SEM. n=6-8.

3.3.3.2 Time-profiling effects of 2'-MeCCPA (10nM) on infarct size to risk ratio within isolated hearts subjected to ischaemia reperfusion injury

All hearts were subjected to 20 minutes stabilisation, 35 minutes of ischaemia and a further 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at the onset of reperfusion and the infarct size to risk ratio was compared to when 2'-MeCCPA (10nM) was administered 15 minutes into the onset of reperfusion as well as 30 minutes into the onset of reperfusion.

A significant decrease in infarct size to risk ratio was detected when 2'-MeCCPA (10nM) was administered at the onset of reperfusion when compared to the IR control (28 ± 4% vs. 55 ± 6%, $p<0.001$). A significant decrease in infarct size to risk ratio was also observed when 2'-MeCCPA (10nM) was also administered at 15 minutes into the onset of reperfusion (30 ± 10%

vs. 55 ± 6%, p<0.001) as well as when 2'-MeCCPA was administered 30 minutes into the onset of reperfusion (34 ± 6% vs. 55 ± 6%, p<0.001) as seen in Figure 3.11.

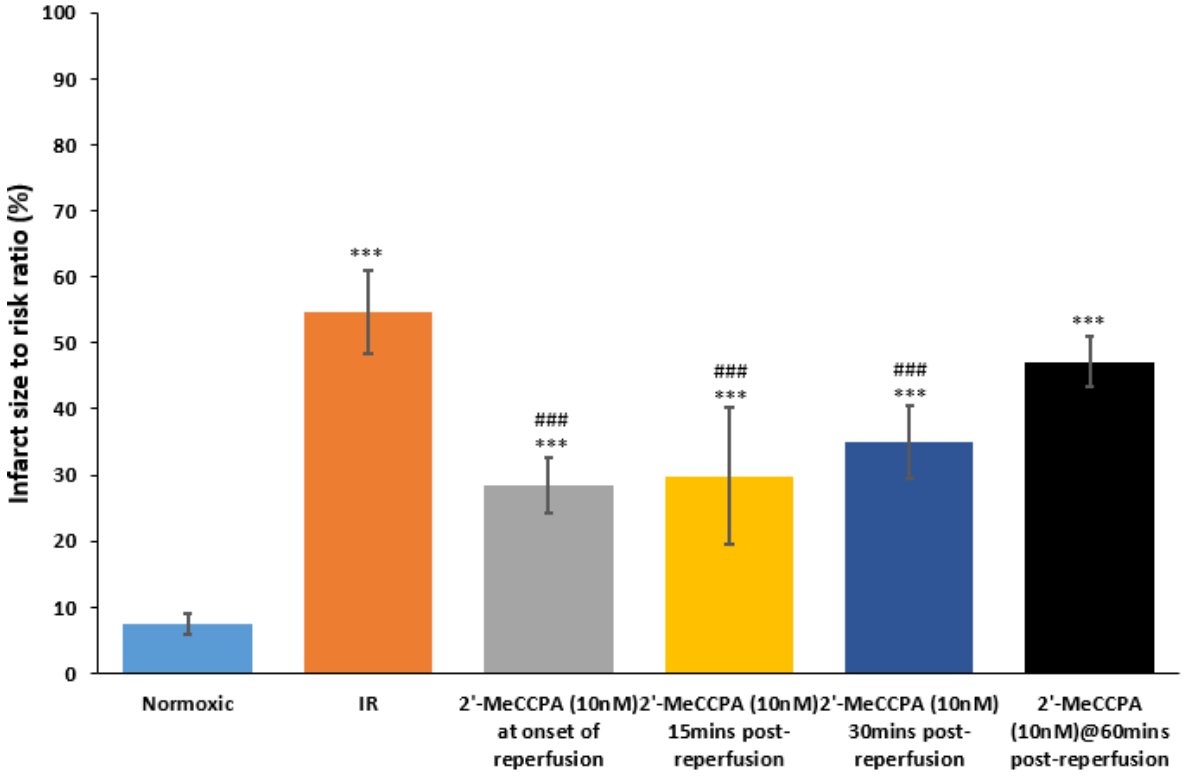


Figure 3. 11 Infarct size to risk ratio (%) within isolated perfused hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence and absence of 2'-MeCCPA (10nM) administered at the onset of reperfusion as well as administered 15 minutes into the onset of reperfusion, 30 minutes into the onset of reperfusion and 60 minutes into the onset of reperfusion. Data presented as Mean±SEM. n=6-8. *** p<0.001 vs. normoxic, ### p<0.001 vs. IR.

3.3.3.3 Time-point profiling effects of 2'-MeCCPA (10nM) when administered at the onset of reoxygenation, 15 minutes post-reoxygenation and 30 minutes post-reoxygenation in adult rat cardiomyocytes subjected to hypoxia-reoxygenation on apoptosis and necrosis.

Isolated adult rat myocytes were exposed to 1 hour of hypoxia and 3 hours of reoxygenation. It was observed that when 2'-MeCCPA (10nM) was administered at the onset of reoxygenation there was a significant decrease in apoptotic cardiomyocytes compared to the Hyp/Reox group (17±4% 2'-MeCCPA at onset of reoxygenation vs. 34±6% Hyp/Reox, p<0.001). A significant decrease in necrotic cardiomyocytes was observed when 2'-MeCCPA (10nM) was

administered at the onset of reoxygenation compared to the Hyp/Reox group (13±2% 2'-MeCCPA at onset of reperfusion vs. 28±7% Hyp/Reox, p<0.001) (Figure 3.12).

When 2'-MeCCPA (10nM) was administered at 15 minutes post-reoxygenation, a significant decrease in apoptotic cardiomyocytes was seen in comparison to the Hyp/Reox group (14±5% 2'-MeCCPA 15mins Post-R vs. 34±6% Hyp/Reox, p<0.001). Similarly, when 2'-MeCCPA (10nM) was administered at 15 minutes post-reoxygenation, a significant decrease in necrotic cardiac myocytes was seen compared to the Hyp/Reox group (16±4% 2'-MeCCPA 15mins Post-R vs. 28±7% Hyp/Reox, p<0.001) (Figure 3.12).

The administration of 2'-MeCCPA (10nM) at 30 minutes post-reoxygenation showed a significant decrease in apoptotic cardiomyocytes in comparison to the Hyp/Reox group (15±5% 2'-MeCCPA 30mins Post-R vs. 34±6% Hyp/Reox, p<0.001). A significant decrease in necrotic cardiomyocytes was observed when 2'-MeCCPA was administered 30 minutes post-reoxygenation in comparison to the Hyp/Reox group (17±3% 2'-MeCCPA 30mins Post-R vs. 28±7% Hyp/Reox, p<0.001) (Figure 3.12).

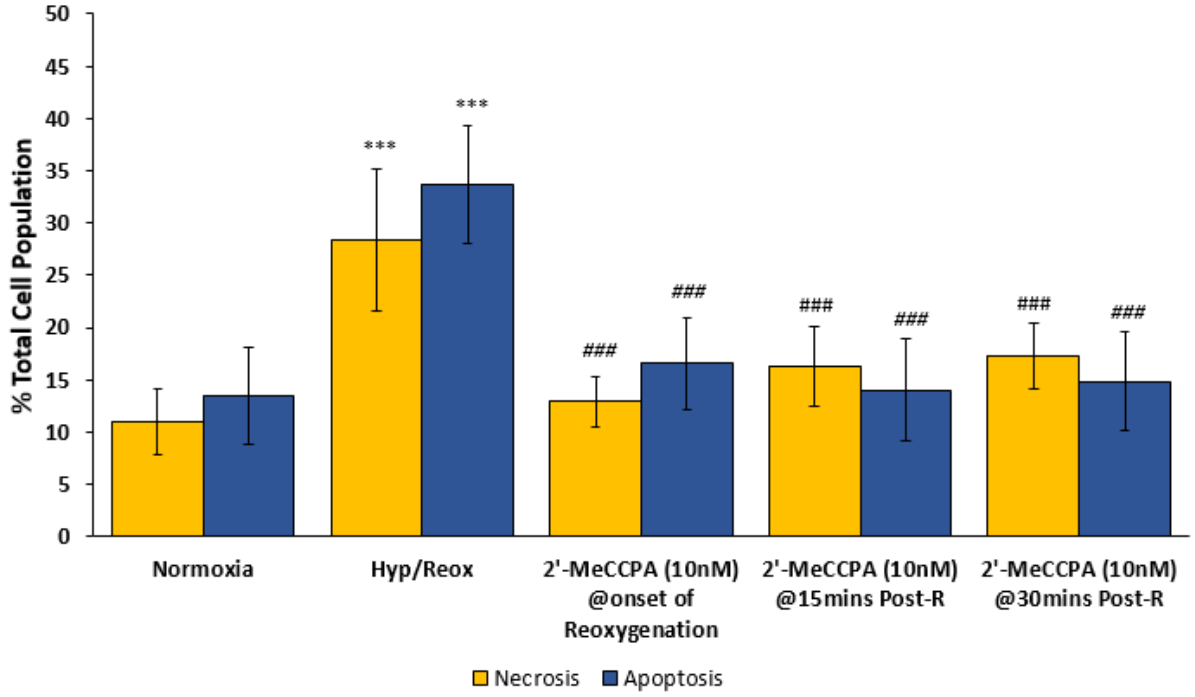


Figure 3. 12 The assessment of apoptosis and necrosis within isolated rat cardiomyocytes subjected to 1 hour hypoxia and 4 hours of reoxygenation. The A_1AR agonist 2'-MeCCPA (10nM) was administered at the onset of reoxygenation, 15 minutes post-reoxygenation and 30 minutes post-reoxygenation. Results are shown as Mean±SEM and are also expressed as a percentage of 10000 cells counted from 6 individual experiments. *** p<0.001 vs. Normoxia. ### p<0.001 vs. Hyp/Reox.

3.3.3.4 Time-point profiling effects of 2'-MeCCPA (10nM) when administered at the onset of reoxygenation, 15 minutes post-reoxygenation and 30 minutes post-reoxygenation on cleaved caspase-3 activity in isolated rat cardiomyocytes when subjected to 1 hour of hypoxia and 3 hours of reoxygenation

Isolated adult rat cardiomyocytes were subjected to 1 hour of hypoxia and 3 hours of reoxygenation. It was observed that when 2'-MeCCPA (10nM) was administered upon the onset of reoxygenation as well as 15 minutes into the onset of reoxygenation, a significant decrease in cleaved-caspase 3 was detected compared to the Hyp/Reox group (181±35% 2'-MeCCPA at the onset of reoxygenation p<0.001, 262±63% 2'-MeCCPA at 15 minutes Post-Reox p<0.05 vs. 313±35% Hyp/Reox) (Figure 3.13). A non-significant decrease in cleaved-caspase 3 activity was detected when 2'-MeCCPA was administered at 30 minutes post-reoxygenation in comparison to Hyp/Reox group (p>0.05) (Figure 3.13).

A significant increase in cleaved-caspase 3 activity was detected when 2'-MeCCPA was administered 15 minutes post-reperfusion compared to when 2'-MeCCPA was administered at the onset of reoxygenation (retrospectively, 262±63% vs 181±35%, $p<0.01$) (Figure 3.13). A significant increase in cleaved-caspase 3 activity was also detected when 2'-MeCCPA was administered at 30 minutes post-reperfusion compared to when 2'-MeCCPA was administered at the onset of reoxygenation (retrospectively, 306±77% vs. 181±35%, $p<0.05$) (Figure 3.13).

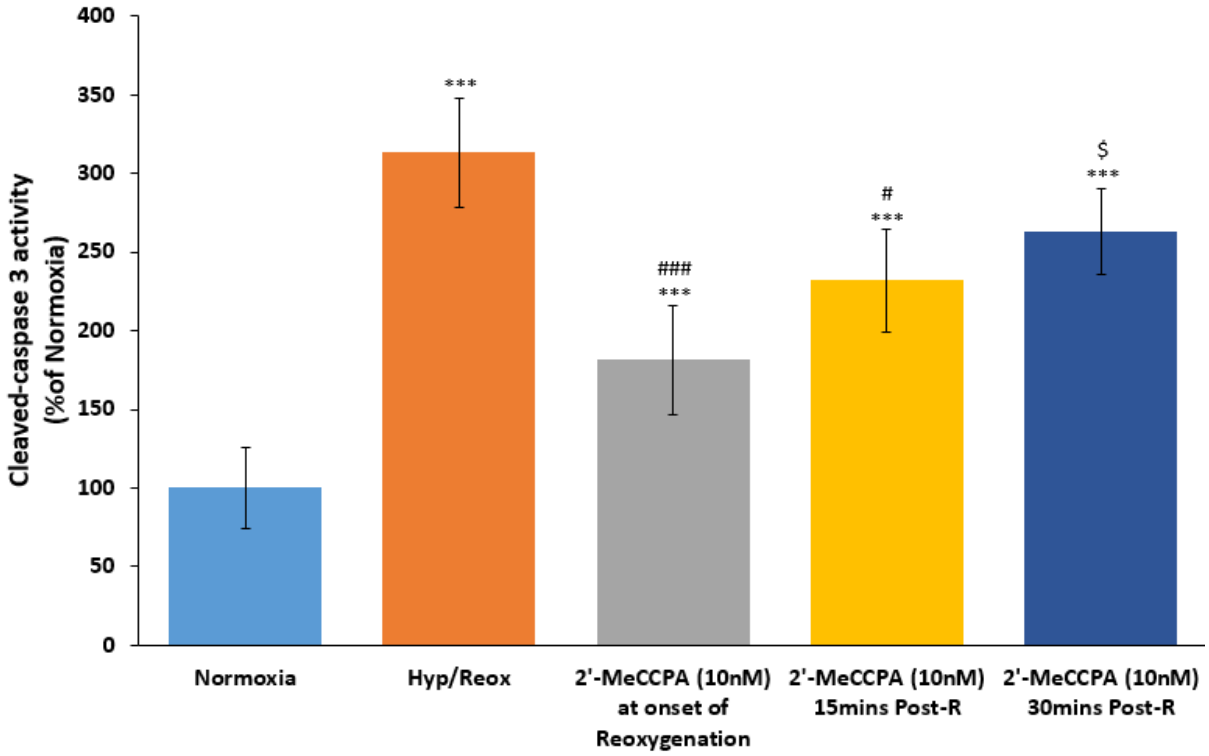


Figure 3. 13 Cleaved-caspase 3 activity in isolated rat cardiomyocytes subjected to 4 hours of reoxygenation (Normoxia) or 1 hour of hypoxia followed by 3 hours of reoxygenation (Hyp/Reox). 2'-MeCCPA (10nM) was administered at the onset of reoxygenation, 15 minutes post-reoxygenation as well as 30 minutes post-reoxygenation. *** $p<0.001$ vs. Normoxia. ### Vs. Hyp/Reox. # $p<0.05$ vs. Hyp/Reox. $$ $p<0.01$ vs. 2'-MeCCPA (10nnM) Onset of Reox. $ $p<0.05$ vs. 2'-MeCCPA (10nM) Onset of Reox. Mean±SEM of 6 experiments.

3.4 Discussion

In this current investigation, we demonstrated that altering the concentration of 2'-MeCCPA from 0.1nM - 1μM within isolated perfused rat heart had little effect upon haemodynamic parameters HR, LVDP and CF except at the concentration of 100nM where 2'-MeCCPA showed a significant decrease in HR after the onset of reperfusion (Figure 3.2.). This is can be supported by published research by Schindler and colleagues (2005) who observed that when the A_1 agonist, CPA (0.3mg/kg) was administered to the rat heart model, a significant decrease in heart rate and blood pressure was observed (Schindler et al. 2005). The findings of the current study can also be compared to a study by Koeppen et al. (2009) where they presented *in vivo* evidence in anaesthetised mice heart models to support how the A_1 adenosine receptor plays a selective role in slowing heart rate (Koeppen et al. 2009). This current investigation only showed the slowing of the HR when 2'-MeCCPA (100nM) was administered at reperfusion, this could potentially be useful within a clinical setting.

The way in which the A_1 adenosine receptor decreases HR is by atrioventricular block (AV) upon activation (Albrecht-Kupper et al. 2012). They inhibit the pacemaker current which decreases the pacemaker action potential and therefore causes negative chronotropy (Mustafa et al. 2009). The A_1 adenosine receptor activation can act upon the presynaptic purinergic receptors that are located on the sympathetic nerve terminals which furthermore inhibit the release of noradrenaline and in turn decrease HR (Mustafa et al. 2009). This could be the effect occurring when 2'-MeCCPA (100nM) is being administered to activate the A_1 adenosine receptors.

When 2'-MeCCPA (100nM) was administered at the onset of reperfusion, a decrease in heart rate was detected however there was no difference to LVDP within this current study, which Schindler and colleagues (2005) had observed. This could have been due to the fact that optimal concentration to affect LVDP may not have been reached and higher concentrations of 2'-MeCCPA could have been investigated and explored.

From this current study, it can be implied that when 2'-MeCCPA (10nM) was administered at 15 minutes and 30 minutes post-reperfusion, there was no significant difference in LVDP which is also surprising. This could be due to the fact that such a low concentration of 2'-

MeCCPA was administered and this could have been investigated using a higher concentration of 2'-MeCCPA rather than 10nM for haemodynamic parameters only.

Although the concentration of 2-MeCCPA (100nM) was observed to slow the HR, this current study observed that the concentration of 2'-MeCCPA (10nM) was shown to decrease infarct size to risk ratio (%) the most compared to the other concentrations of 2'-MeCCPA (0.1nM, 1nM, 100nM and 1μM); and therefore taken forward within this study for further investigation. The lower concentration of 2'-MeCCPA (10nM) was further used as it provided the most significant amount of protection to the isolated rat heart and rat cardiomyocyte models. Using such a low concentration of 2'-MeCCPA (10nM) ensured that infarct size to risk ratio (%), cell death and caspase-3 activity data remained significantly less. One of the main purposes of this current study was to investigate the cardioprotective effects of the A_1 adenosine receptor when activated and this aim was achieved.

The concentration dependent effects of 2'-MeCCPA (0.1nM - 1μM) were assessed on the isolated rat heart model to further determine the effects on infarct size to risk ratio. This method of investigation was useful and can be compared to the methodology conducted by Maddock et al. (2002) where they also studied the concentration dependent effects of the A_3 adenosine receptor agonist 2-CI-IB-MECA within the isolated rat heart model upon infarct size to risk ratio (%) (Maddock et al. 2002). With similar methodology used to assess an A_3 adenosine receptor agonist, it was only beneficial to apply it when investigating an A_1 adenosine receptor agonist such as 2'-MeCCPA.

This current investigation can imply that the A_1 adenosine receptor agonist 2'-MeCCPA between the concentrations of 1nM - 1μM were able to confer cardioprotection within the ischaemia reperfusion rat heart model via a decrease in infarct size to risk ratio (%). This is useful and can be compared to previous literature that have also used heart models within rabbits, canine and mouse to confer cardioprotection (Baxter et al. 2000; Yao and Gross 1993; Urmaliya et al. 2010). Baxter et al. (2000) showed that when an A_1 adenosine receptor agonist, GR79236 (30 μg kg^{-1}), was administered to an ischaemic reperfused myocardium within the rabbit heart model, it limited lethal reperfusion injury via infarct size (Baxter et al. 2000). Yao and Gross (1993) also suggested that the stimulation of the myocardial A_1 adenosine receptors with A_1AR agonist CPA, within an ischaemia reperfusion model of a stunned canine model, had the ability to confer cardioprotection through repetitive but brief periods of

coronary artery occlusion (Yao and Gross 1993). A more recent study by Urmaliya et al. (2010) showed that when A_1AR agonist CPA (100nM) was administered to wild-type mouse cardiac cell ischaemia model that were subjected to global ischaemia, there was a significant reduction in infarct size due to the activation of the A_1 adenosine receptor. An interesting point from this study is that 100nM concentration was used and if this was administered to mice, the effects on haemodynamic parameters could have been assessed and compared to our current study. A further study by Bibli and colleagues (2014) showed that the A_1 adenosine receptor activation with CCPA in rabbit heart is essential for triggering cardioprotection through the post-conditioning phenomenon and on this basis, our current study can imply that the activation of A_1 adenosine receptors with 2'-MeCCPA (10nM) in rat heart can trigger cardioprotection through the post-reperfusion activation phenomenon.

Due to the fact that various concentrations of A_1AR agonist 2'-MeCCPA (0.1nM - 1µM) were assessed in the isolated rat heart model with the Langendorff technique, the same assessment was carried out in isolated rat ventricular cardiomyocytes to investigate cell death by apoptosis and necrosis as well cleaved caspase-3 activity. These elements were investigated to imply which concentration limited cell death and cleaved caspase-3 activity. Assessment of these markers is important as they allow for the determination of the cardioprotective effects of 2'-MeCCPA on a cellular level.

This current study found that when concentrations of 2'-MeCCPA (1nM - 1µM) were administered to isolated rat cardiomyocytes, there was an overall decrease in apoptosis and necrosis of cardiomyocytes at all nanomolar concentrations. This meant that receptor activation of the A_1 adenosine receptors has shown to decrease apoptotic injury that has been induced by ischaemia. This can be linked with previously published data by Maddock et al. (2002) that researched to imply that the activation of the A_3 adenosine receptor also had the ability to decrease apoptotic injury at nanomolar concentrations of CL-IB-MECA. Although an A_3 adenosine agonist was investigated by Maddock et al (2002), it did imply that lower concentrations of the A_3 adenosine agonist had an effect upon apoptotic injury and this could also be similar with the A_1 adenosine activation; and from our findings, it was.

With limited published research conducted into the A_1 adenosine receptor through post-reperfusion activation, further research has suggested that the A_3 adenosine receptor functions in a similar manner to the A_1 adenosine receptor to mediate cardioprotection

(Safran et al. 2001; Liang et al. 2001, Mangoni 2004), therefore similarities could potentially be drawn upon due to the way in which both receptor subtypes are activated. Both the A_1 and the A_3 adenosine receptor subtypes are coupled to the G_i and G_o pertussis toxin sensitive proteins which makes them different to the A_{2A} and A_{2B} adenosine receptor subtypes (Germack and Dickenson 2004; Hoffman et al. 1997).

This current study implied that the A_1 adenosine receptor agonist 2'-MeCCPA had the ability to decrease cleaved caspase-3 activity between the concentrations of 1nM – 100nM which decreased cardiomyocyte apoptosis. Current literature has not been able to clearly decipher the link between the activation of A_1 adenosine receptors and a decrease in cleaved caspase-3 within the cardiovascular system through the post-reperfusion activation phenomenon however it has been in the pre-conditioning phenomenon (Germack and Dickenson 2005). Interestingly Dastjerdi et al. (2016) have shown that the expression of the A_1 adenosine receptor within breast tissue was able to significantly down-regulate caspase-3 activity within MCF-7 cells and when A_1 adenosine receptor antagonist DPCPX was administered, the down-regulation of caspase-3 was reversed. This therefore implies that the activation of the A_1 adenosine receptors does play a part in the decrease in cleaved caspase-3 activity.

Apoptosis is divided into 2 distinct signalling pathways which include the intrinsic pathway (also known as the mitochondrial cell death pathway) and the extrinsic pathway (also known as the death receptor pathway) (Fulda 2010; Kim and Kang 2010). Many investigations have shown that upon the activation of either the intrinsic or extrinsic pathway, there are initiator caspases that become activated that go on to trigger executioner caspases -3, -6 and -7 (Singh and Kang 2011; Zhang et al 2017). Within this current study, when 2'-MeCCPA (1nM - 1µM) was administered to isolated rat cardiomyocytes to activate the A_1 adenosine receptor subtype, it was shown that apoptosis was decreased however cleaved caspase-3 activity was also decreased. The activation of caspase-3 is linked with mitochondrial oxidative stress which can also lead to an increase in cytochrome c release into the cytosol thus producing apoptosome complexes (Eefting et al. 2004; Gottlieb et al. 1994; Wan and Yim 2005). Due to the decrease in apoptosis with 2'-MeCCPA (1nM - 1µM) administration, this also meant a lack of mitochondrial oxidative stress and therefore a lack of apoptosome complex formation. This implies that the A_1 adenosine receptor agonist, 2'-MeCCPA possesses cardioprotective qualities at a cellular level. Further study by Germack and colleagues (2004; 2005) showed by

A_1 activation can cause protection to neonatal rat cardiomyocytes via the upregulation of the MEK/ERK1/1 and PI3K-AKT signalling pathways causing the decrease in caspase-3 activity and therefore a decrease in apoptosis.

Previous research has shown that the activation of the A_1 adenosine receptor prior to ischaemia (known as pre-conditioning) within the rabbit and rat model has expressed cardioprotective effects (Hill et al. 1998; Hochhauser et al. 2007). There has, however, been little, if any, research on activating A_1 adenosine receptors post-reperfusion to determine their cardioprotective effects. Postponing the administration of cardioprotective agents after the onset of reperfusion is still considered as a novel hypothesis and is still being extensively researched. Data from this current study implies that activation of the A_1 adenosine receptors using selective A_1 adenosine agonist, 2'-MeCCPA (10nM), and administering it at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion all have the ability to mediate cardioprotection in the form of reducing infarct size to risk ratio in the isolated rat model. This post-reperfusion administration also has the ability to decrease apoptosis and necrosis as well as caspase-3 activity at different time-points. An article written my Piper, Abdallah and Schäfer (2004) implied that the first few minutes of reperfusion constituted as a critical phase in which lethal tissue injury can occur and therefore this window of opportunity can lead to cardioprotection (Piper, Abdallah and Schäfer 2004). This therefore supports the findings of this current study when 2'-MeCCPA (10nM) has been administered at the onset of reperfusion/reoxygenation and at 15 minutes post-reperfusion/reoxygenation to provide a cardioprotective effect. Interestingly, this study also implies that the cardioprotective effect is also observed at 30 minutes post-reperfusion/reoxygenation which has never been detected before. This could be useful within a clinical setting as 30 minutes after an ischaemic myocardial event, the A_1 adenosine receptor agonist 2'-MeCCPA could provide cardioprotective effects.

This study demonstrated and therefore implied that postponing the administration of 2'-MeCCPA to 15 minutes and 30 minutes post-reperfusion still conferred cardioprotection via the reduction of infarct size in isolated perfused rat hearts. Postponing the administration of this cardioprotective agent until after the onset of reperfusion has not actually been extensively researched. Interestingly, Von Lubitz et al. (2001) showed how the A_3AR agonist IB-MECA, when administered at 20 minutes post-reperfusion, successfully attenuated infarct

development in mice brain that were subjected to ischaemia-reperfusion. This post ischaemic treatment resulted in an improved neuronal preservation and therefore protection of neuronal activity in mice brain (Von Lubitz et al. 2001). This technique of post-reperfusion activation of the A_3 adenosine receptor was very interesting and applied to this current project into the post-reperfusion activation of the A_1 adenosine receptor and found that protective effects were observed.

3.5 Summary of Findings

In summary, the present study represents the evidence that the A_1 adenosine receptor agonist, 2'-MeCCPA, may be able to reduce myocardial ischaemia reperfusion injury in Sprague Dawley rat model. Our results show that:

- Activation of the A_1 adenosine receptor at the onset of reperfusion using various concentrations of selective A_1 adenosine receptor agonist, 2'-MeCCPA (1nM, 10nM, 100nM and 1µM), offers cardioprotection.
- When the A_1 adenosine receptor is activated using selective A_1AR agonist 2'-MeCCPA (1nM, 10nM, 100nM and 1µM), there was a reduction in infarct size to risk ratio (%) observed in ischaemic-reperfused myocardium.
- The administration of 2'-MeCCPA (1nM, 10nM, 100nM and 1µM) to rat ventricular cardiomyocytes caused a decrease in apoptosis and necrosis which in turn decreased caspase-3 activity, an effector enzyme of the apoptotic cell death cascade.
- The activation of A_1 adenosine receptors using the selective A_1AR agonist 2'-MeCCPA (10nM) when administered at 15 minutes and 30 minutes post-reperfusion conferred cardioprotection.
- The administration of the A_1AR agonist 2'-MeCCPA (10nM) at 15 minutes and 30 minutes post-reperfusion caused a decrease in infarct size to risk ratio (%) in ischaemic-reperfused myocardium.
- The administration of A_1AR agonist 2'-MeCCPA (10nM) to isolated rat ventricular cardiomyocytes at 15 minutes and 30 minutes post-reperfusion both causes a decrease in apoptosis and necrosis which also caused a decrease in caspase-3 activity.

Chapter 4: Adenosine Antagonist Profiling with A_1 Adenosine Receptor (A_1AR) Agonist alongside Selective A_1 and Non-Selective Adenosine Antagonists at Reperfusion and Post-Reperfusion

4.1 Introduction

Considerable research has been conducted into the four different adenosine receptors (A_1, A_{2A}, A_{2B} and A_3). They have been cloned and characterised within several mammalian species. Adenosine itself is the preferred endogenous agonist that targets all four different receptor subtypes. Adenosine levels throughout an injurious period such as ischaemia has the ability to activate all receptors, even when expressed at low levels (Fredholm et al. 2001; Covinhes et al. 2020). Previous studies have investigated receptor antagonism within knock out mice to investigate adenosine A_1, A_2 and A_3 receptor expression and how they respond under physiological and pathophysiological conditions (Fredholm et al. 2001). Previous research has been conducted into these receptor subtypes where selective agonists have been used to activate certain receptor subtypes and selective antagonist have been used in order to identify the specific role of that receptor. Both agonists and antagonist are being developed as potential drug therapies. This current study explores the role of the A_1 adenosine receptor and if there is a potential interaction from other adenosine receptor subtypes for cardioprotection.

Numerous studies have explored the protective qualities of adenosine in myocardial reperfusion injury (Yao and Gross 1993) and have shown that the administration of adenosine can limit infarct size (Yao and Gross 1993). There have also been many studies that have investigated the activation of the A_1 adenosine receptor through the phenomenon of preconditioning to enhance cardioprotection in animal models such as rat neonatal myocytes and canine models (Germack and Dickenson 2004; Yao and Gross 1993). Further research is required in order to elucidate exact activation of the A_1 adenosine receptor throughout post-reperfusion activation and if alternative adenosine receptor subtypes could potentially play a part towards the cardioprotection expressed in rat myocardium.

The role of an antagonist is to obstruct the preferred effect of an agonist. A very common and well documented A_1 adenosine antagonist, DPCPX, has been administered alongside many

selective A_1 adenosine agonists in order to block the effect of the A_1AR agonist. Urmaliya et al. (2010) administered DPCPX alongside A_1AR agonist CPA causing successful attenuation of CPA-mediated cardioprotection. This allowed for the confirmation of the A_1 receptor to play an important part in cardioprotection.

4.1.1. Aims and Objectives

1. The aim of this investigation was to determine the explicit effects of A_1 adenosine receptor activation at the onset of reperfusion as well as delayed receptor activation of the A_1 adenosine receptor at 15 minutes and 30 minutes post-reperfusion by activation with A_1 adenosine receptor agonist 2'-MeCCPA (10nM) and then blocking the A_1 adenosine receptor subtype with the co-administration with A_1 adenosine antagonist DPCPX (200nM) within the isolated perfused rat heart model and isolated rat cardiomyocyte model. Effects on infarct size (%), apoptosis, necrosis and cleaved caspase-3 activity were assessed.
2. The second aim of this investigation was to determine the effects of p-AKT $_{(Ser473)}$ phosphorylation when 2'-MeCCPA was administered in the presence and absence of A_1AR antagonist DPCPX at the onset of reperfusion, 15 minutes or 30 minutes post-reperfusion through western blot analysis. This was carried out to determine the explicit effects of the A_1 adenosine receptor towards the regulation of the PI3K-AKT and pro-survival pathway.
3. Another aim of this investigation was to assess the effects of A_1 adenosine receptor activation at the onset of reperfusion but also as delayed receptor activation at 15 minutes and 30 minutes post-reperfusion by the administration of the A_1 adenosine agonist 2'-MeCCPA (10nM). 2'-MeCCPA (10nM) was then co-administered alongside non-selective adenosine antagonist 8-SPT (1μM) to assess the effects upon infarct size (%), apoptosis, necrosis and cleaved caspase-3 activity. This investigation was carried out to assess the effects upon the explicit activation of the A_1 adenosine receptor subtype and if potentially there is another receptor enhancing cardioprotection.

4.2 Methods

4.2.1 Chemicals

2'-MeCCPA and DPCPX were both purchased from Tocris Cookson (Bristol, UK). 8-SPT was purchased from Carbosynth (Berkshire, UK). Working concentrations of the drug used within this chapter were freshly prepared each day as described in Chapter 2, Section 2.2.

4.2.1 Animals

Adult male Sprague-Dawley rats (350 ± 50g) were sacrificed via cervical dislocation in accordance to the Schedule 1 Home Office Procedure using the process of thoracotomy, previously described in Chapter 2, Section 2.1.

4.2.3 Langendorff protocol - Isolated perfused rat heart preparation

For studies involving the Langendorff model of ischaemia-reperfusion (details stated in Chapter 2, Section 2.3.5), all experiments were carried out for 175 minutes. Isolated rat hearts were given 20 minutes for stabilisation followed by 35 minutes of simulated regional ischaemia and 120 minutes of reperfusion. Hearts were random allocated to the following control and treatment groups:

a) Normoxic control – Rat hearts perfused with KH buffer for 175 minutes (no ischaemia was induced).
b) Ischaemia-Reperfusion (IR) Control – Rats hearts were perfused with KH buffer for 20 minutes followed by 35 minutes of simulation regional ischaemia and then 120 minutes of reperfusion.
c) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of A_1AR antagonist DPCPX (200nM) at the onset of reperfusion - Rats were perfused with KH buffer for 20 minutes, 35 minutes of simulated regional ischaemia followed by 120 minutes of reperfusion where drug administration occurred at the onset of reperfusion.
d) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of A_1AR antagonist DPCPX (200nM) at 15 minutes post-reperfusion - Rats were perfused with

KH buffer for 20 minutes, 35 minutes of simulated regional ischaemia followed by 120 minutes of reperfusion where drug administration occurred at 15 minutes post-reperfusion.

e) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of A_1AR antagonist DPCPX (200nM) at 30 minutes post-reperfusion - Rats were perfused with KH buffer for 20 minutes, 35 minutes of simulated regional ischaemia followed by 120 minutes of reperfusion where drug administration occurred at 30 minutes post-reperfusion.

f) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of non-selective adenosine antagonist 8-SPT (1µM) at the onset of reperfusion - Rats were perfused with KH buffer for 20 minutes, 35 minutes of simulated regional ischaemia followed by 120 minutes of reperfusion where drug administration occurred at the onset of reperfusion.

g) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of non-selective adenosine antagonist 8-SPT (1µM) at 15 minutes post-reperfusion - Rats were perfused with KH buffer for 20 minutes, 35 minutes of simulated regional ischaemia followed by 120 minutes of reperfusion where drug administration occurred at 15 minutes post-reperfusion.

h) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of non-selective adenosine antagonist 8-SPT (1µM) at 30 minutes post-reperfusion - Rats were perfused with KH buffer for 20 minutes, 35 minutes of simulated regional ischaemia followed by 120 minutes of reperfusion where drug administration occurred at 30 minutes post-reperfusion.

Throughout all Langendorff experiments, the stability of the hearts were consistently monitored through haemodynamic parameters such as: heart rate (HR), left ventricular developed pressure (LVDP) and coronary flow (CF) (as described in Chapter 2, Section 2.3.2).

After the reperfusion period, the infarct size to risk ratio was assessed with the use of Evans blue triphenyltetrazolium chloride (TTC) staining procedures as described in Chapter 2, Section 2.3.6.

4.2.4 Isolation of adult rat ventricular cardiomyocytes

Isolation of adult rat ventricular cardiomyocytes was previously described in Chapter 2, Section 2.4.

4.2.5 Induction of hypoxia and reoxygenation conditions in adult rat cardiomyocytes

Fully described in Chapter 2, Section 2.4.1.

4.2.6 Experimental drug treatment protocol in adult rat ventricular cardiomyocytes

The isolated rat cardiomyocytes were exposed to differing control and drug treatments. All experimental conditions are detailed as below:

a) Normoxic control – Isolated myocytes were exposed to normoxic conditions for a total of 4 hours at 37^{o}C, 5% CO_2 and 95% O_2.
b) Hypoxia-Reoxygenation control – Isolated cardiomyocytes were exposed to 1 hour of hypoxia conditions followed by the onset of reoxygenation for 3 hours.
c) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of A_1AR antagonist DPCPX (200nM) at the onset of reoxygenation - Isolated cardiomyocytes were exposed to 1 hour hypoxia conditions followed by drug treatment at the onset of reoxygenation for 3 hours at 37^{o}C, 5% CO_2 and 95% O_2.
d) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of A_1AR antagonist DPCPX (200nM) at 15 minutes post-reoxygenation - Isolated cardiomyocytes were exposed to 1 hour hypoxia conditions followed by drug treatment at 15 minutes post-reoxygenation for 3 hours at 37^{o}C, 5% CO_2 and 95% O_2.
e) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of A_1AR antagonist DPCPX (200nM) at 30 minutes post-reoxygenation - Isolated cardiomyocytes were exposed to 1 hour hypoxia conditions followed by drug treatment at 30 minutes post-reoxygenation for 3 hours at 37^{o}C, 5% CO_2 and 95% O_2.

f) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of non-selective adenosine antagonist 8-SPT (1µM) at the onset of reoxygenation - Isolated cardiomyocytes were exposed to 1 hour hypoxia conditions followed by drug treatment at the onset of reoxygenation for 3 hours at 37°C, 5% CO_2 and 95% O_2.

g) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of non-selective adenosine antagonist 8-SPT (1µM) at 15 minutes post-reoxygenation - Isolated cardiomyocytes were exposed to 1 hour hypoxia conditions followed by drug treatment at 15 minutes post-reoxygenation for 3 hours at 37°C, 5% CO_2 and 95% O_2.

h) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of non-selective adenosine antagonist 8-SPT (1µM) at 30 minutes post-reoxygenation - Isolated cardiomyocytes were exposed to 1 hour hypoxia conditions followed by drug treatment at 30 minutes post-reoxygenation for 3 hours at 37°C, 5% CO_2 and 95% O_2.

4.2.7 Quantitative Analysis of Cell Death using Dead Cell Apoptosis Kit with Annexin V FITC with Propidium Iodide (PI) in Adult Rat Cardiomyocytes

Followed by the drug treatments described above, isolated rat ventricular cardiomyocytes were assess for their levels of apoptosis and necrosis using the Dead Cell Apoptosis Kit with Annexin V FITC and PI was purchased from ThermoFisher (UK) (previously detailed in Chapter 2, Section 2.5.2). Data was normalised against the cell only control and the values obtained were calculated as a relative change in apoptosis and necrosis activity of the mean absorbance of the control group.

4.2.8 Analysis of Cleaved Caspase-3 Activity in Adult Rat Cardiomyocytes

Following the drug treatment protocol as described above, the rat cardiomyocytes were probed with cleaved caspase-3 antibody as described in Chapter 2, Section 2.5.1. Data obtained was normalised to the cell only control by subtracting the mean fluorescence background recorded in untreated samples. Data was presented as a relative change in fluorescence activity.

4.2.9 Western blot analysis

Western blot analysis was performed as described in Chapter 2, Section 2.6. Following the stages of separation and protein transfer procedure; membrane blots were probed for phosphorylated and total form of the monoclonal rabbit AKT $_{(Ser473)}$ protein (Cell Signalling, UK) as stated in Section 2.6.7. Protein detection was quantified with the use of Super Signal West Femto Maximum Substrate Solution (ThermoFisher Scientific, UK) to perform an enhanced chemiluminescence assay (as detailed in Chapter 2, Section 2.6.8) and proteins bands were visualised with the use of Bio-Rad Quantity One programme. The relative variations of the levels of phosphorylated monoclonal rabbit AKT $_{(Ser473)}$ protein were normalised to the total form of the monoclonal rabbit AKT protein. GAPDH was also used as an internal loading control within all experiments as explained in Chapter 2, Section 2.6.7.

4.2.10 Data Analysis

All data that was presented in this project is expressed at the mean ± standard error of the mean (SEM). IBM Statistical Package for Social Sciences (SPSS®) software was used to statistically analyse the data. The statistical tests currently used to analyse infarct sizes, band densities and cell population data was by one-way ANOVA accompanied by Fishers Protected Least Significant Difference (LSD) test for multiple comparisons. To assess the difference in the data sets, a p-value of $p<0.05$ was used to consider statistical significance.

Microsoft Excel was also used to present all data graphically.

4.3 Results

4.3.1 The effects of co-administration of 2'-MeCCPA (10nM) with A_1 adenosine antagonist, DPCPX (200nM) at various time-points within reperfusion in myocardial ischaemia reperfusion injury.

4.3.1.1 The co-administration effects of 2'-MeCCPA (10nM) + DPCPX (200nM) when administered at the onset of reperfusion on haemodynamic parameters (left ventricular developed pressure, heart rate and coronary flow).

All hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia followed by 120 minutes of reperfusion where A_1 adenosine receptor agonist, 2'-MeCCPA (10nM), was administered in conjunction with A_1 adenosine receptor antagonist, DPCPX (200nM). This co-administration was conducted at the onset of reperfusion within this section. Overall, it was found that there was no significant difference between the groups at any of the time-points within the reperfusion period ($p>0.05$) (Figure 4.1).

Throughout the ischaemic period, all treatment groups had a significantly decreased LVDP in comparison to the normoxic control ($p<0.05$) (Figure 4.1).

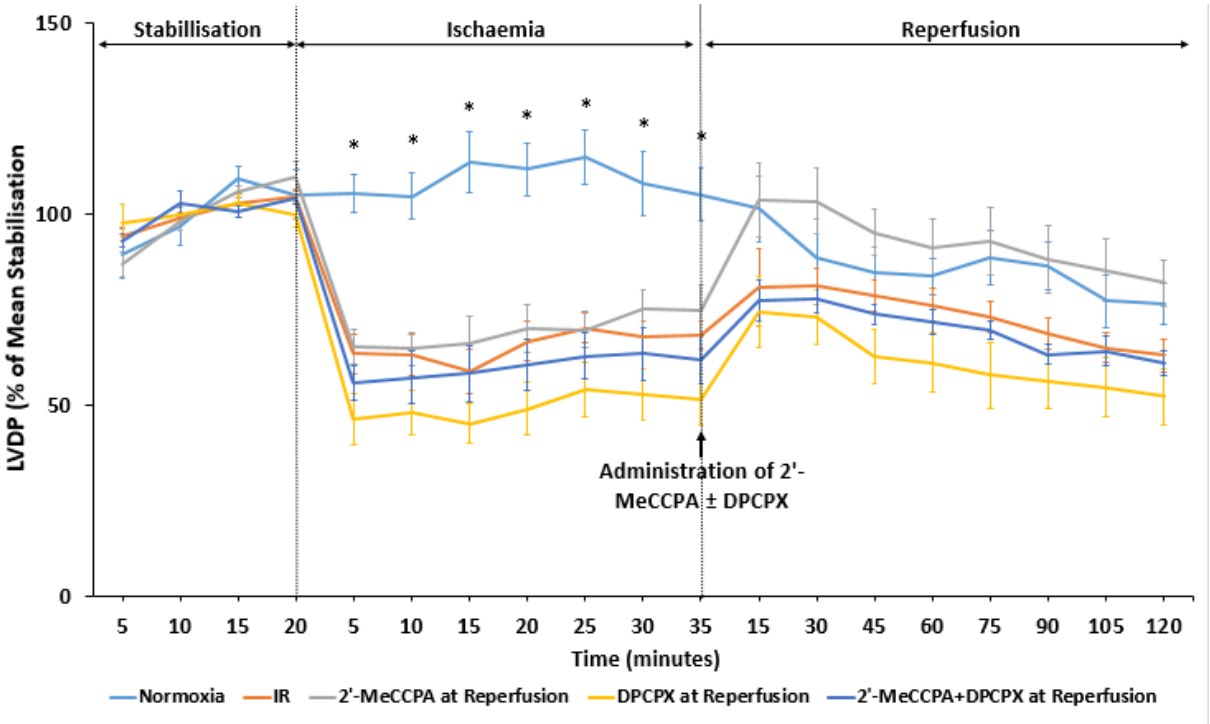


Figure 4. 1 Effects of 2'-MeCCPA (10nM) in the presence and absence of DPCPX (200nM) on the left ventricular developed pressure within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at the onset of reperfusion in the presence and absence of DPCPX (200nM). Data was presented as Mean±SEM, n=6-8. * $p<0.05$ vs. Normoxia.

The co-administration of 2'-MeCCPA (10nM) in the presence and absence of DPCPX (200nM) had no significant difference upon heart rate at all time-points post reperfusion when compared to the IR control ($p>0.05$) (Figure 4.2).

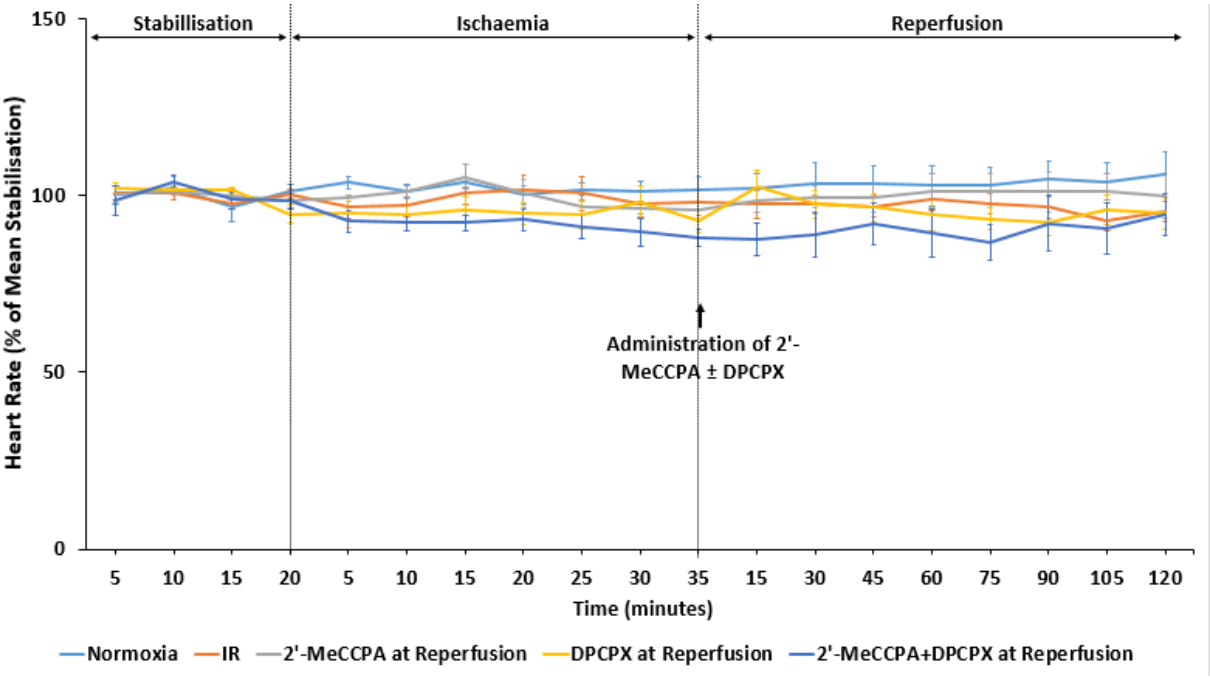


Figure 4. 2 The effects of 2'-MeCCPA (10nM) in the presence and absence of DPCPX (200nM) on heart rate within isolated rat hearts subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at the onset of reperfusion in the presence and absence of DPCPX (200nM). Data was presented as Mean±SEM. n=6-8.

2’-MeCCPA (10nM) when administered in the presence of absence of DPCPX (200nM) has no significant effect upon coronary flow when compared to the IR control hearts post-reperfusion (p>0.05, Figure 4.3).

Throughout the ischaemic period, all treatment groups had a significantly decreased coronary flow in comparison to the normoxic control (p<0.05) (Figure 4.3).

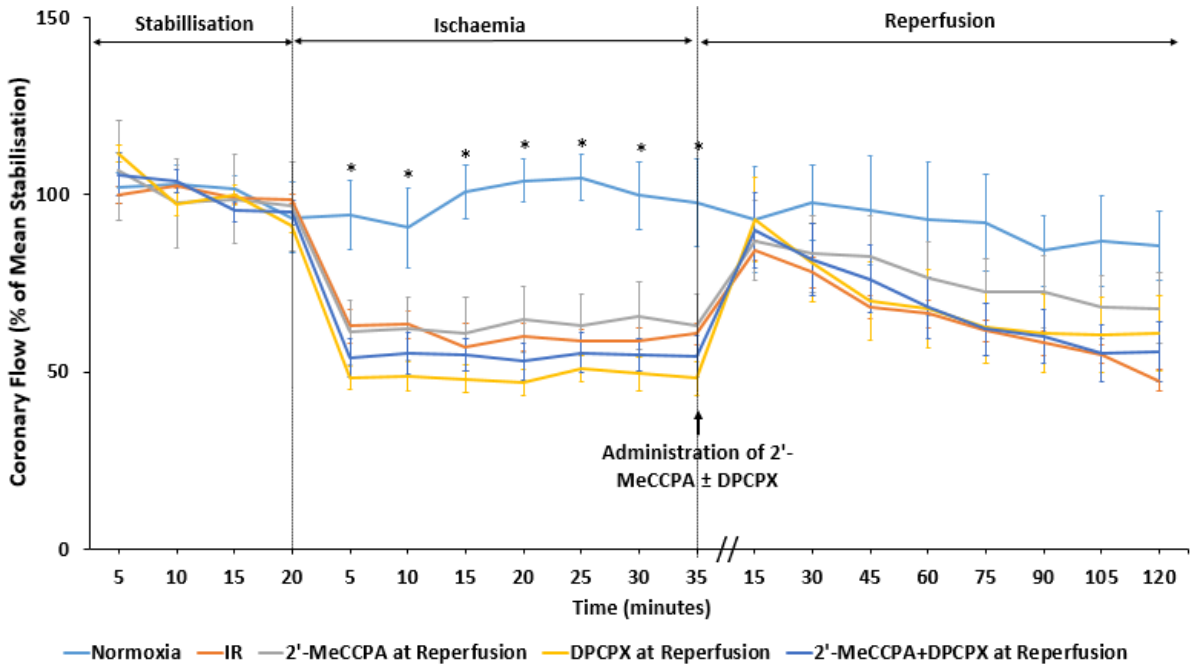


Figure 4. 3 Effects of 2’-MeCCPA (10nM) administered in the presence and absence of DPCPX (200nM) on coronary flow in isolated rat hearts subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2’-MeCCPA (10nM) was administered at the onset of reperfusion in the presence and absence of DPXPC (200nM). Data was presented as Mean±SEM. n=6-8. * p<0.05 vs. Normoxia.

4.3.1.2 The effects of co-administration of 2'-MeCCPA (10nM) with A_1 adenosine antagonist, DPCPX (200nM) on infarct size to risk ratio within isolated hearts subjected to ischaemia reperfusion injury at the onset of reperfusion.

A significant decrease in infarct size to risk ratio (%) was detected when 2'-MeCCPA (10nM) was administered at the onset of reperfusion compared to the IR control (28 ± 4% vs. 55 ± 6%, p<0.001). When 2'-MeCCPA (10nM) + DPCPX (200nM) was administered together at the onset of reperfusion, a significant increase in infarct size to risk ratio (%) was detected when compared to the administration of 2'-MeCCPA (10nM) alone (52 ± 7% vs. 28 ± 4%, p<0.001) (Figure 4.4).

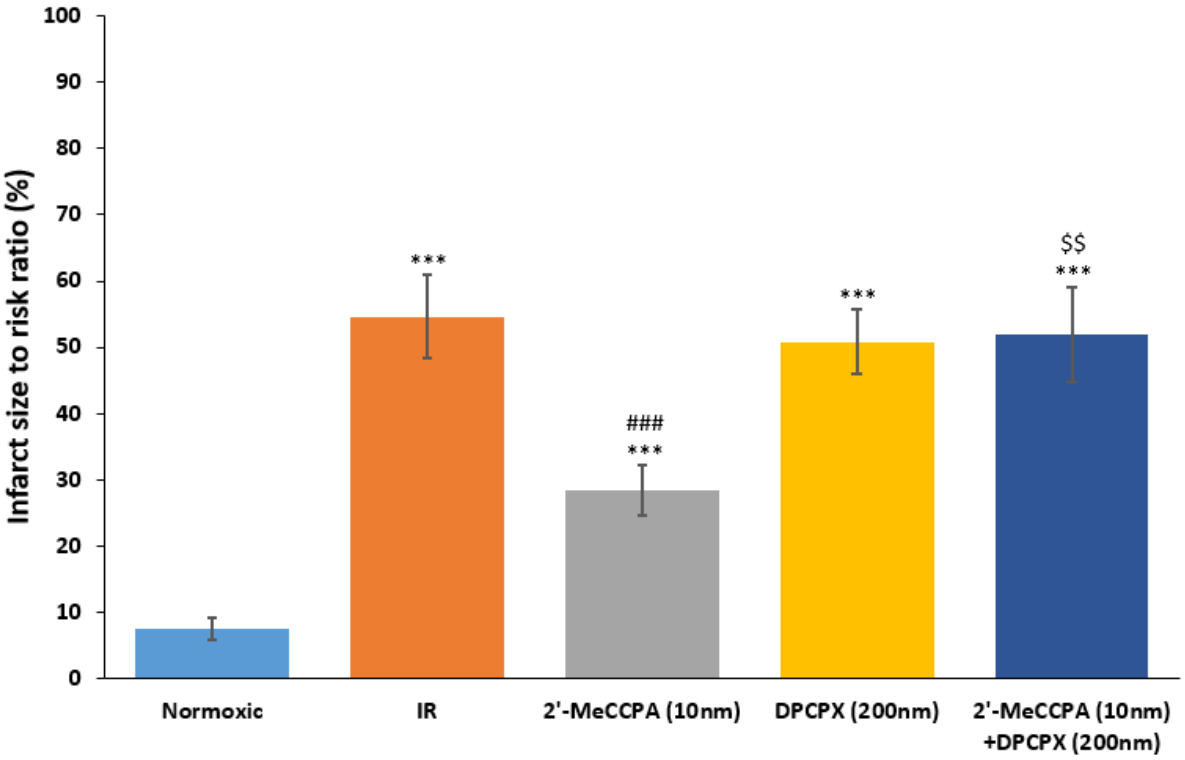


Figure 4. 4 Infarct size to risk ratio (%) within isolated perfused rats' hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Hearts were subjected to 2'-MeCCPA (10nM) administered at the onset of reperfusion in the presence and absence of DPCPX (200nM). Data presented as Mean±SEM. n=6-8. *** p<0.001 vs. normoxic, ### p<0.001 vs. IR and $$ p<0.01 vs. 2'-MeCCPA (10nM).

4.3.1.3 The co-administration effects of 2'-MeCCPA (10nM) + DPCPX (200nM) when administered at 15 minutes post-reperfusion on haemodynamic parameters (left ventricular developed pressure, heart rate and coronary flow).

All hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia followed by 120 minutes of reperfusion where A_1 adenosine receptor agonist, 2'-MeCCPA (10nM), was administered in conjunction with A_1 adenosine receptor antagonist, DPCPX (200nM). This co-administration was administered 15 minutes pot-reperfusion within this section. Overall, it was found that there was no significant difference between the groups at any of the time-points ($p>0.05$).

The co-administration of 2'-MeCCPA (10nM) in conjunction with DPCPX (200nM) had no significant effect upon LVDP at all time-points post reperfusion when compared to the IR control ($p<0.05$) (Figure 4.5).

Throughout the ischaemic period, all treatment groups had a significantly decreased LVDP in comparison to the normoxic control ($p<0.05$) (Figure 4.5).

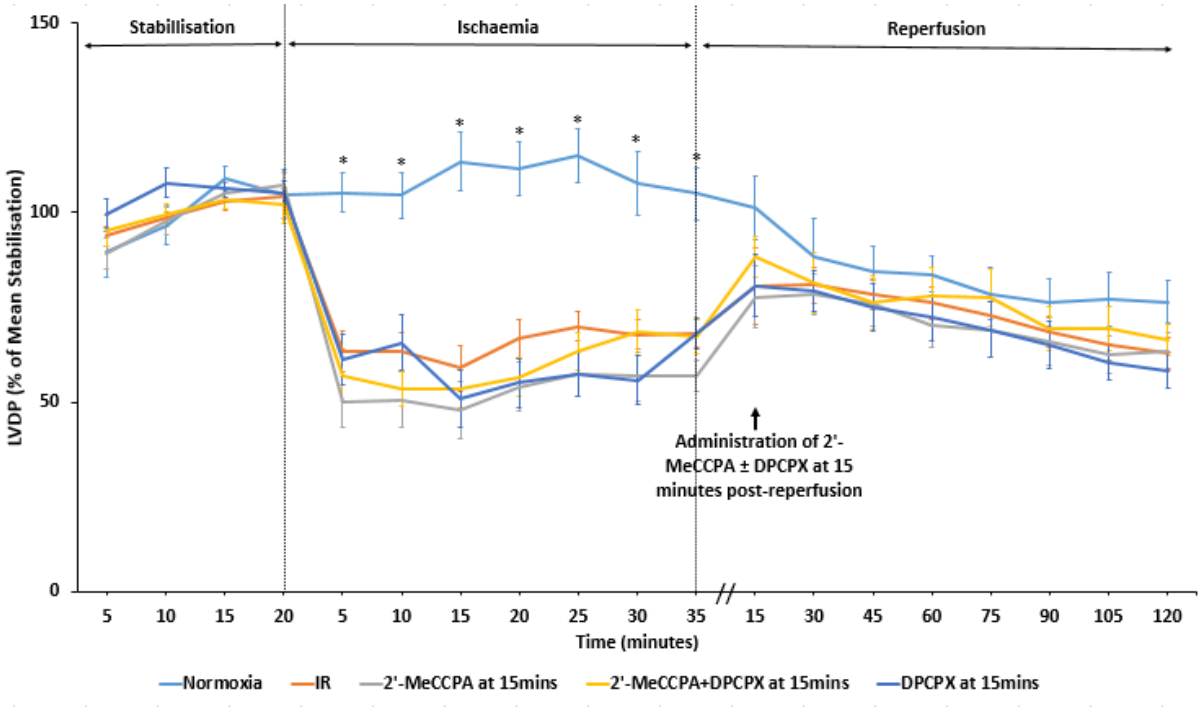


Figure 4. 5 Effects of 2'-MeCCPA (10nM) administered in the presence and absence of DPCPX (200nM) on left ventricular developed pressure within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at 15 minutes post-reperfusion in the presence and absence of DPCPX (200nM). Data was presented as Mean±SEM. n=6-8. * $p<0.05$ vs. Normoxia.

When 2'-MeCCPA (10nM) and DPCPX (200nM) were administered together at 15 minutes post-reperfusion, no significant difference was detected upon heart rate at all time-points post reperfusion in comparison to the IR control (Figure 4.6).

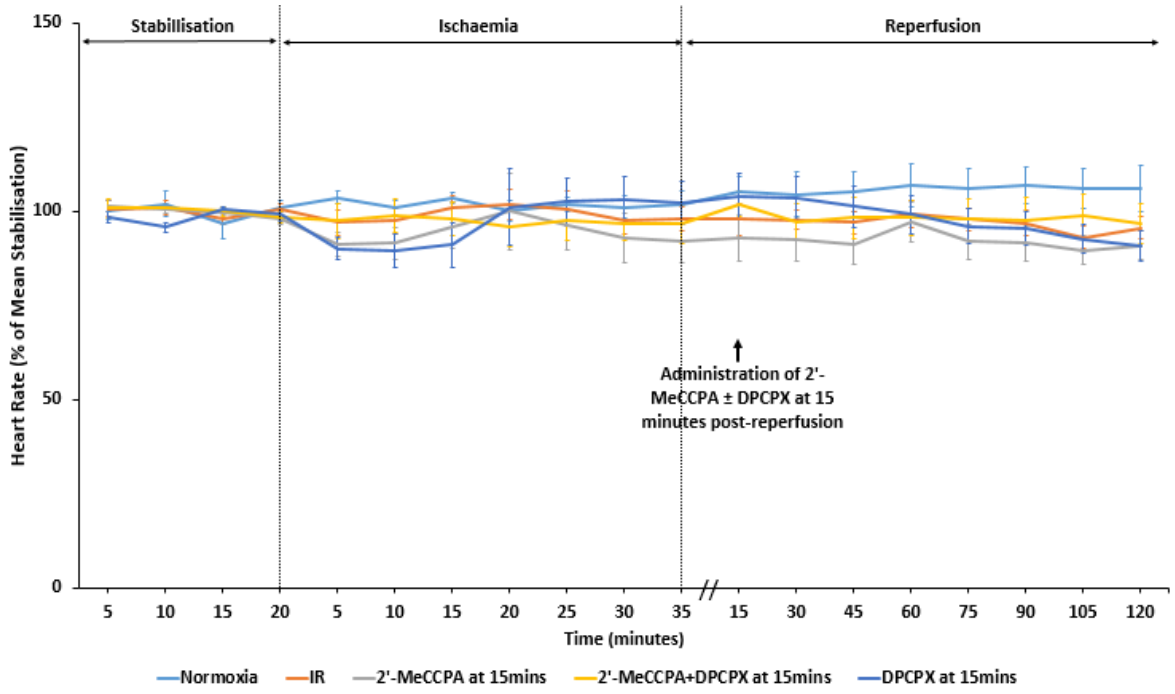


Figure 4. 6 Effects of 2'-MeCCPA (10nM) administered in the presence and absence of DPCPX (200nM) on heart rate within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and a further 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at 15 minutes post-reperfusion in the presence and absence of DPCPX (200nM). Data was presented at Mean±SEM. n=6-8).

When 2'-MeCCPA (10nm) and DPCPX (200nM) was administered in conjunction, no significant difference was detected on coronary flow at all time points within the reperfusion period ($p<0.05$) (Figure 4.7).

Throughout the ischaemic period, all treatment groups had a significantly decreased coronary flow in comparison to the normoxic control ($p<0.05$) (Figure 4.7).

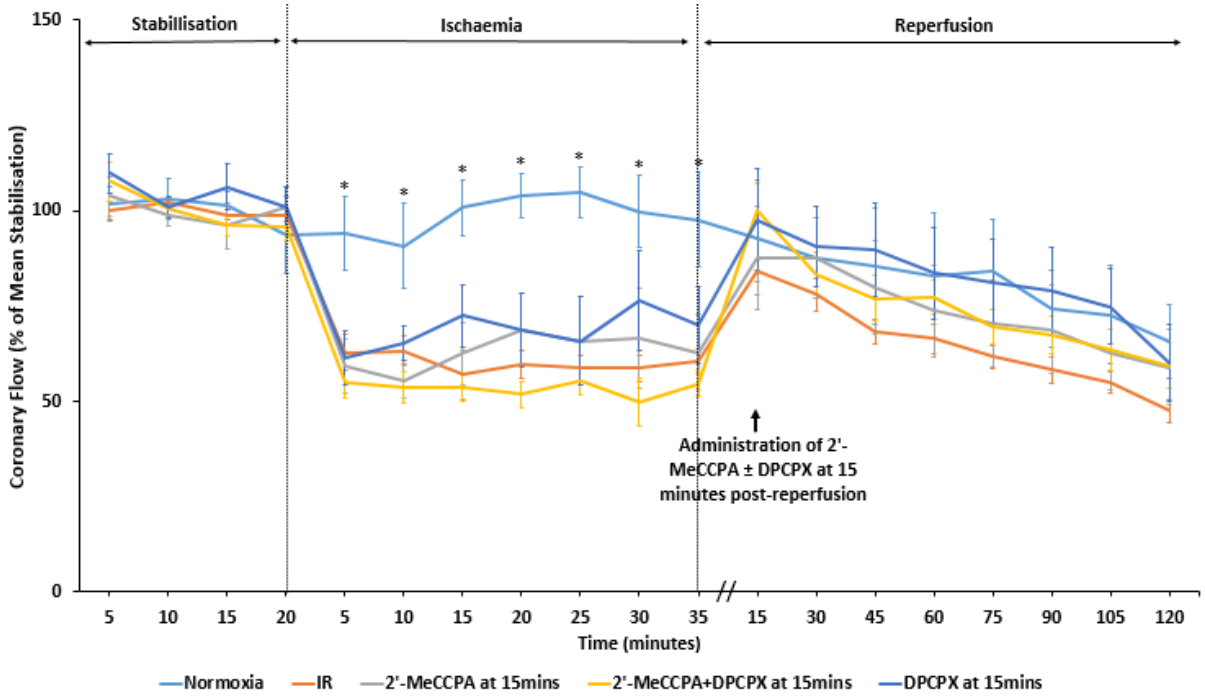


Figure 4. 7 Effects of 2'-MeCCPA (10nM) administered in the presence and absence of DPCPX (200nM) o heart rate within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at 15 minutes post-reperfusion in the presence and absence of DPCPX (200nM). Data was presented at Mean±SEM. n=6-8. * $p<0.05$ vs. Normoxia.

4.3.1.4 The effects of co-administration of 2'-MeCCPA (10nM) with A_1 adenosine antagonist, DPCPX (200nM) on infarct size to risk ratio in isolated hearts subjected to ischaemia reperfusion injury at 15 minutes post-reperfusion

A significant decrease in infarct size to risk ratio (%) was observed when 2'-MeCCPA (10nM) alone was administered at 15 minutes post-reperfusion in comparison to the IR control (30 ± 10% vs. 55 ± 6%, $p<0.001$) (Figure 4.8). When 2'-MeCCPA (10nM) + DPCPX (200nM) was

administered in conjunction with one another at 15 minutes post-reperfusion, a significant increase in infarct size to risk ratio (%) was detected in comparison to when 2'-MeCCPA (10nM) was administered alone at 15 minutes post-reperfusion (56 ± 7% vs. 30 ± 10%, p<0.01) (Figure 4.8). Administration of DPCPX had no significant effect upon infarct size to risk ratio (%) when compared to the IR control.

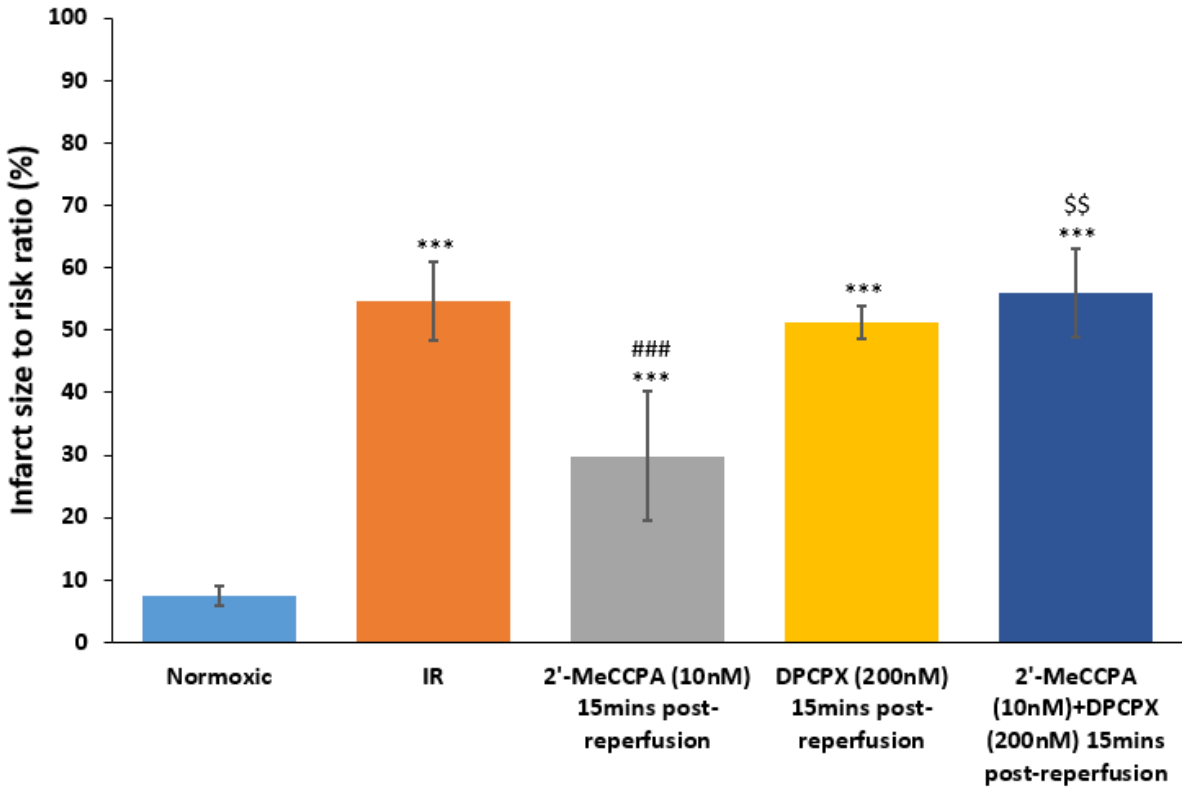


Figure 4. 8 Infarct size to risk ratio (%) within isolated perfused hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion subjected to 2'-MeCCPA (10nM) administered at 15 minutes post-reperfusion alone as well as DPCPX (200nM) administered at 15 minutes post-reperfusion alone and the co-administration of 2'-MeCCPA (10nM) + DPCPX (200nM) administered at 15 minutes post-reperfusion. Data presented as Mean±SEM. n=6-8. *** p<0.001 vs. normoxic, ### p<0.001 vs. IR and $$ p<0.01 vs. 2'-MeCCPA administered 15 minutes post-reperfusion (10nM).

4.3.1.5 The co-administration effects of 2'-MeCCPA (10nM) + DPCPX (200nM) when administered at 30 minutes post-reperfusion on haemodynamic parameters (left ventricular developed pressure, heart rate and coronary flow).

Hearts were all subjected to a 20 minute stabilisation period, 35 minutes of ischaemia followed by 120 minutes of reperfusion where A_1 adenosine receptor agonist, 2'-MeCCPA (10nM) was administered in conjunction with A_1 adenosine receptor antagonist, DPCPX (200nM). 2'-MeCCPA was administered alone, DPCPX was administered alone and both were also co-administered together at 30 minutes post-reperfusion.

The co-administration of 2'-MeCCPA in the presence and absence of DPCPX at 30 minutes post-reperfusion had no significant difference between the groups at any of the time-points within the reperfusion period ($p>0.05$) (Figure 4.9).

Throughout the ischaemic period, all treatment groups had a significantly decreased LVDP in comparison to the normoxic control ($p<0.05$) (Figure 4.9).

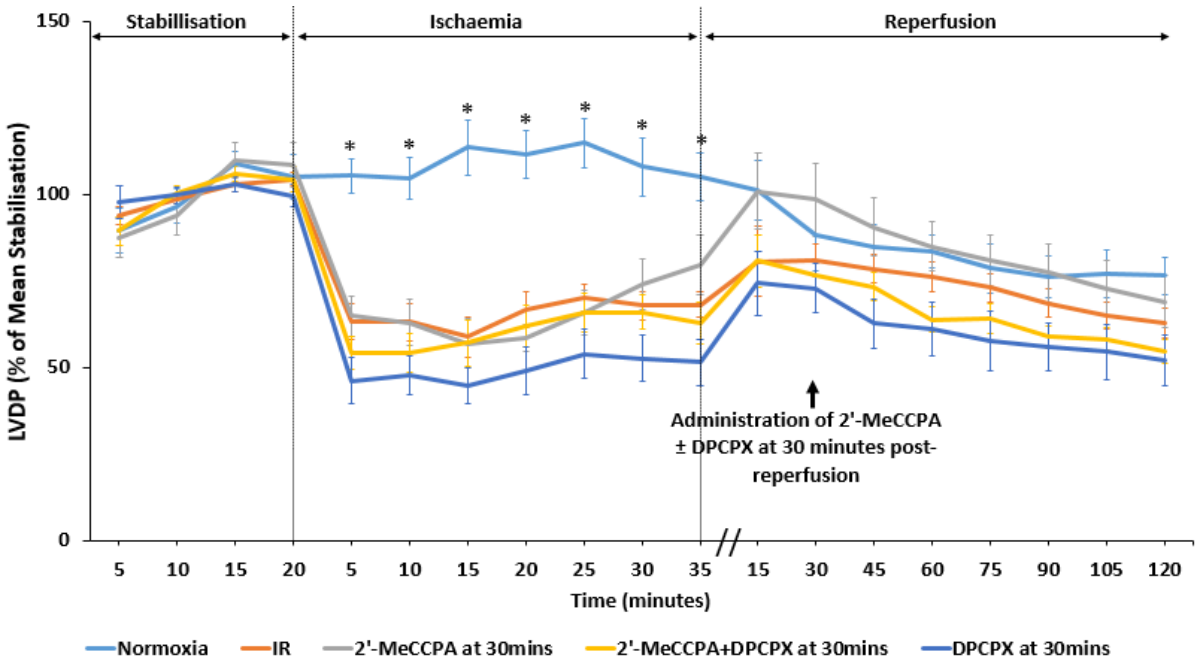


Figure 4. 9 Effects of 2'-MeCCPA (10nM) administered in the presence and absence of DPCPX (200nM) on left ventricular developed pressure within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at 30 minutes post-reperfusion in the presence and absence of DPCPX (200nM). Data was presented as Mean±SEM. n=6-8. * $p<0.05$ vs. Normoxia.

No significant effect was detected upon heart rate when 2'-MeCCPA and DPCPX was administered in conjunction at 30 minutes post-reperfusion at any time-points in comparison to IR control and normoxic control ($p>0.05$) (Figure 4.10).

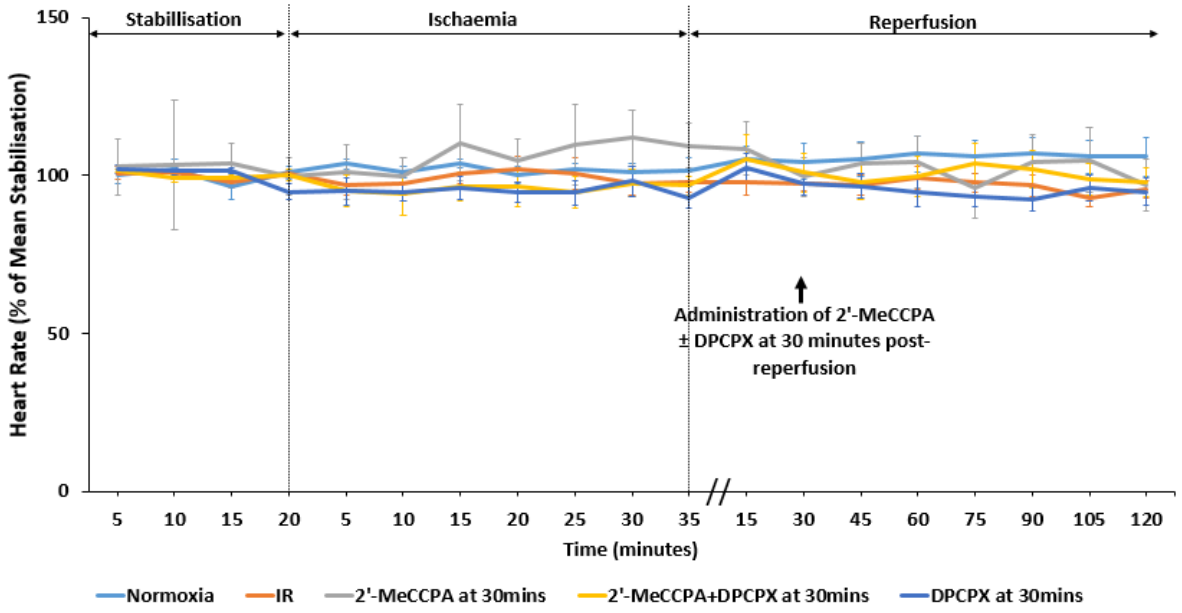


Figure 4. 10 Effects of 2'-MeCCPA (10nM) administered in the presence and absence of DPCPX (200nM) on heart rate within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and a further 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at 30 minutes post-reperfusion in the presence and absence of DPCPX (200nM). Data was presented at Mean±SEM. n=6-8.

When 2'-MeCCPA (10nM) was administered alongside DPCPX (200nM) as well as when each of these treatments were administered separately too, there was no significant difference detected between groups and when compared to the IR control within the reperfusion period ($p>0.05$) (Figure 4.11).

Throughout the ischaemic period, all treatment groups had a significantly decreased coronary flow in comparison to the normoxic control ($p<0.05$) (Figure 4.11).

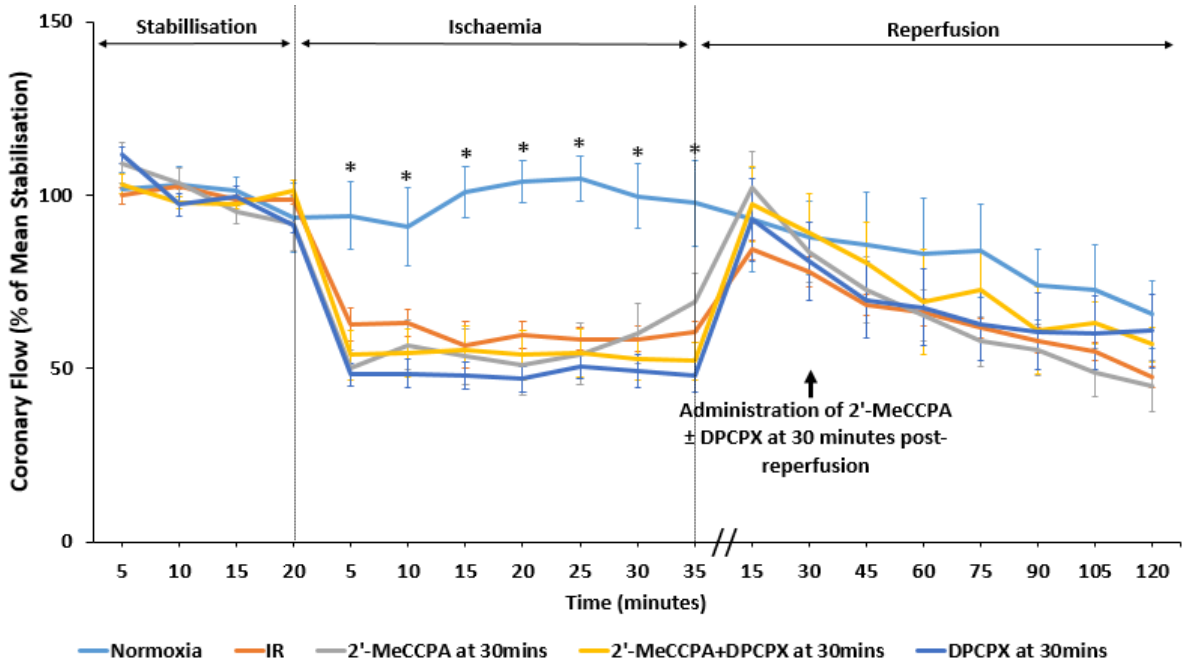


Figure 4. 11 Effects of 2'-MeCCPA (10nM) administered in the presence and absence of DPCPX (200nM) on heart rate within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at 30 minutes post-reperfusion in the presence and absence of DPCPX (200nM). Data was presented at Mean±SEM. n=6-8. * $p<0.05$ vs. Normoxia.

4.3.1.6 Effects of co-administration of 2'-MeCCPA (10nM) with A_1 adenosine antagonist, DPCPX (200nM) on infarct size to risk ratio within isolated hearts subjected to ischaemia reperfusion injury at 30 minutes post-reperfusion.

When 2'-MeCCPA (10nM) was administered alone at 30 minutes post reperfusion, a significant decrease in infarct size to risk ratio (%) was detected when compared to the IR control (35 ± 6% vs. 55 ± 6%, $p<0.001$) (Figure 4.12). When DPCPX (200nM) was administered alone at 30 minutes post-reperfusion, no significant effect was observed. When 2'-MeCCPA (10nM) + DPCPX (200nM) were administered together at 30 minutes post-reperfusion and a

significant increase in infarct size to risk ratio was observed when comparing to when 2'-MeCCPA was administered alone at 30 minutes post-reperfusion (57 ± 11% vs. 35 ± 6%, $p<0.05$) (Figure 4.12).

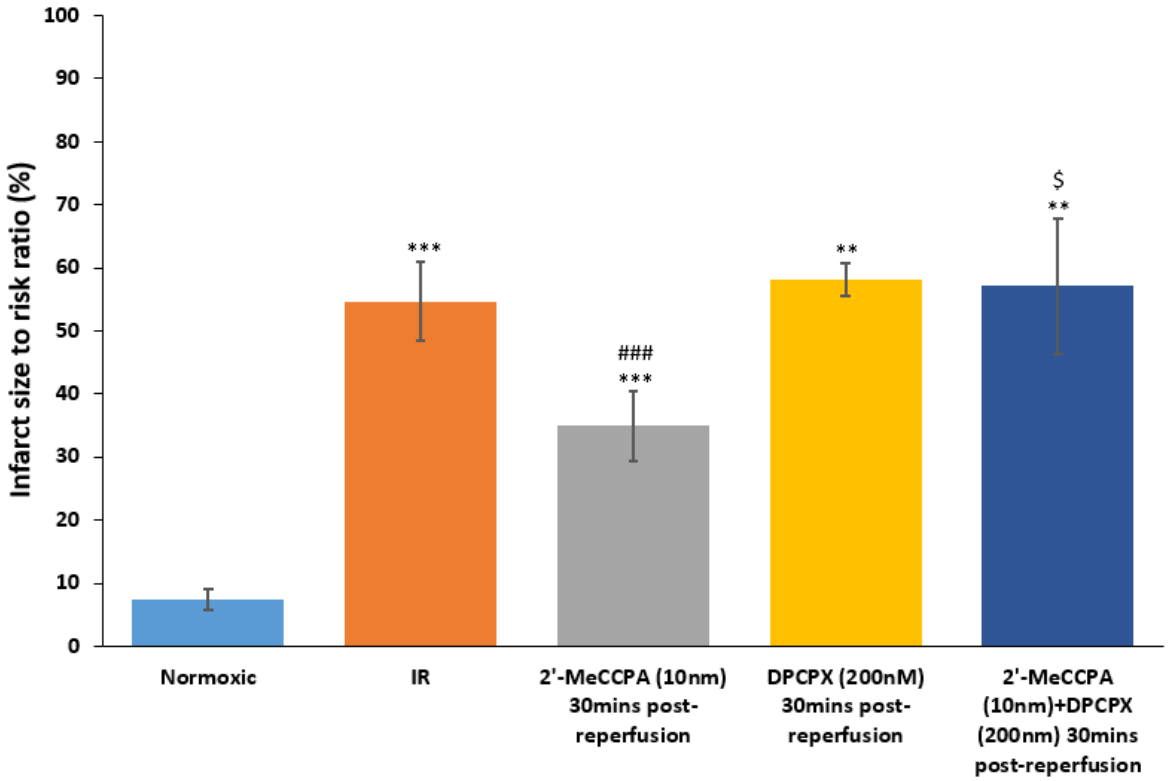


Figure 4. 12 Infarct size to risk ratio (%) within isolated perfused hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion subjected to 2'-MeCCPA (10nM) administered at 30 minutes post-reperfusion alone as well as DPCPX (200nM) administered at 30 minutes post-reperfusion alone and the co-administration of 2'-MeCCPA (10nM) + DPCPX (200nM) administered at 30 minutes post-reperfusion. Data presented as Mean±SEM. n=6-8. *** $p<0.001$ vs. normoxic, ** $p<0.01$ vs normoxic ### $p<0.001$ vs. IR and $ $p<0.05$ vs. 2'-MeCCPA administered 30 minutes post-reperfusion (10nM).

4.3.1.7 Profiling effects of co-administration of 2'-MeCCPA (10nM) alongside A_1 adenosine antagonist, DPCPX (200nM) at the onset of reoxygenation, 15 minutes post-reoxygenation and 30 minutes post-reoxygenation in adult rat cardiomyocytes subjected to hypoxia-reoxygenation on apoptosis and necrosis

Isolated adult rat cardiac myocytes were subjected to 1 hour of hypoxia and 3 hours of reoxygenation and treated with 2'-MeCCPA (10nM) in the presence and absence of DPCPX (200nM). Cardiomyocytes were then assessed for apoptosis and necrosis.

When 2'-MeCCPA (10nM) + DPCPX (200nM) was administered in conjunction at the onset of reoxygenation, there was an increase in cellular apoptosis compared to when 2'-MeCCPA (10nM) was administered alone however this was not a significant change (21±4% 2'-MeCCPA + DPCPX at onset of Reox vs. 17±4% 2'-MeCCPA@onset of Reox, $p<0.05$) (Figure 4.13). When DPCPX (200nM) was administered alone at the onset of reoxygenation, an increase in apoptosis was detected when compared to the non-treated Hyp/Reox group however this was not significant ($p>0.05$) (Figure 4.3). An increase in apoptotic cells was detected when DPCPX (200nM) was administered alone in comparison to when 2'-MeCCPA (10nM) was administered alone, this was not a significant change (Figure 4.13).

When 2'-MeCCPA (10nM) + DPCPX (200nM) was co-administered at the onset of reoxygenation, a significant increase in necrosis was observed when compared to 2'-MeCCPA (10nM) being administered alone (22±6% 2'-MeCCPA + DPCPX vs. 13±2% 2'-MeCCPA, $p<0.01$) (Figure 4.13). A decrease in necrotic cells was observed when DPCPX (200nM) was administered alone at reoxygenation in comparison to the non-treated Hyp/Reox group ($p>0.05$) (Figure 4.13).

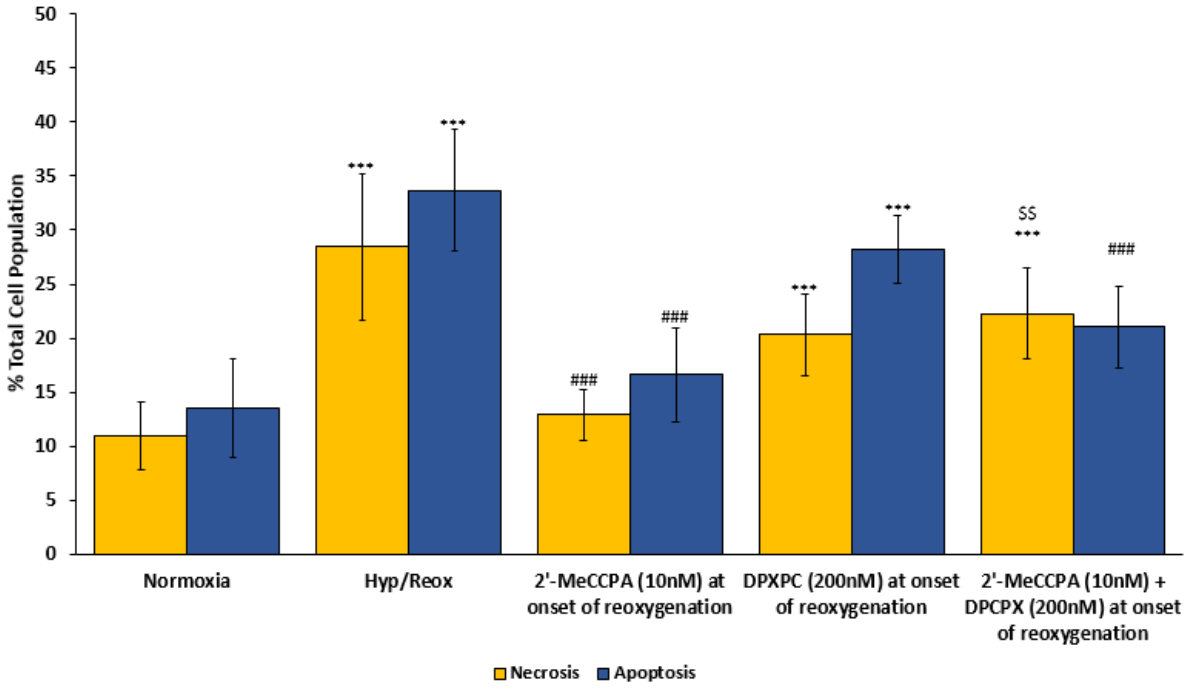


Figure 4. 13 The assessment of necrosis and apoptosis within isolated rat cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation. 2'-MeCCPA (10nM) was administered at the onset of reoxygenation in the presence and absence of A_1 adenosine receptor antagonist, DPCPX (200nM) b. Results were shown as Mean±SEM and were expressed as a percentage of 10 000 cells counted from 6 individual experiments. *** p<0.001 vs. Normoxia. ### p<0.001 vs. Hyp/Reox. $$ p<0.01 vs. 2'MeCCPA at onset of Reox.

A significant increase in cellular apoptosis was also detected when 2'-MeCCPA (10nM) + DPCPX (200nM) was administered together at 15 minutes post-reoxygenation compared to when 2'-MeCCPA (10nM) was administered alone at 15 minutes post-reoxygenation (25±3% 2'-MeCCPA + DPCPX@15mins Post-R vs. 14±5% 2'-MeCCPA@15min Post-R, p<0.01) (Figure 4.14). An increase in cellular apoptosis was detected when DPCPX (200nM) was administered alone at 15 minutes post-reperfusion in comparison to when 2'-MeCCPA (10nM) was administered alone, this was not a significant effect (p<0.05) (Figure 4.14).

When 2'-MeCCPA (10nM) + DPCPX (200nM) was administered in conjunction at 15 minutes post-reoxygenation an increase in cellular necrosis was observed in comparison to when 2'-MeCCPA (10nM) was administered alone at 15 minutes post-reoxygenation, this was not a significant change (21±4% 2'-MeCCPA + DPCPX@15mins Post-R vs. 16±4% 2'-MeCCPA@15mins Post-R, p<0.05) (Figure 4.14).

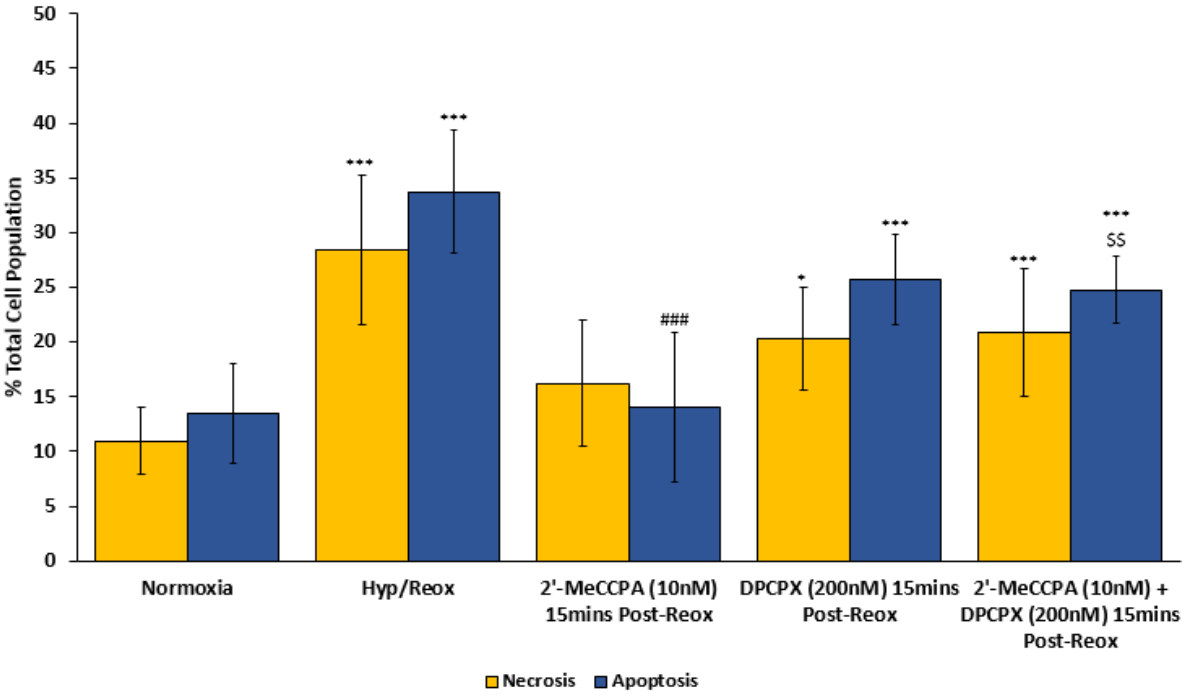


Figure 4. 14 The assessment of necrosis and apoptosis within isolated rat cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation. 2'-MeCCPA (10nM) was administered at 15 minutes post-reoxygenation in the presence and absence of A_1 adenosine receptor antagonist, DPCPX (200nM). Results were shown as Mean±SEM and were expressed as a percentage of 10 000 cells counted from 6 individual experiments. *** $p<0.001$ vs. Normoxia. * $p<0.05$ vs. Normoxia. ### $p<0.001$ vs. Hyp/Reox. $$ $p<0.01$ vs. 2'-MeCCPA 15mins Post-Reox.

When 2'-MeCCPA (10nM) + DPCPX (200nM) was administered together at 30 minutes post-reoxygenation, an increase in cellular apoptosis was detected when compared to when 2'-MeCCPA (10nM) was administered alone at 30 minutes post-reoxygenation however this was not a significant change (21±7% 2'-MeCCPA +DPCPX@30min Post-R vs. 15±6% 2'-MeCCPA@30mins Post-R, $p<0.05$) (Figure 4.15).

When 2'-MeCCPA (10nM) + DPCPX (200nM) was administered together at 30 minutes post-reoxygenation, a significant increase in cellular necrosis was detected when compared to when 2'-MeCCPA (10nM) was administered alone at 30 minutes post-reoxygenation (23±7% 2'-MeCCPA +DPCPX@30min Post-R vs. 17±6% 2'-MeCCPA@30mins Post-R, $p<0.01$) (Figure 4.15).

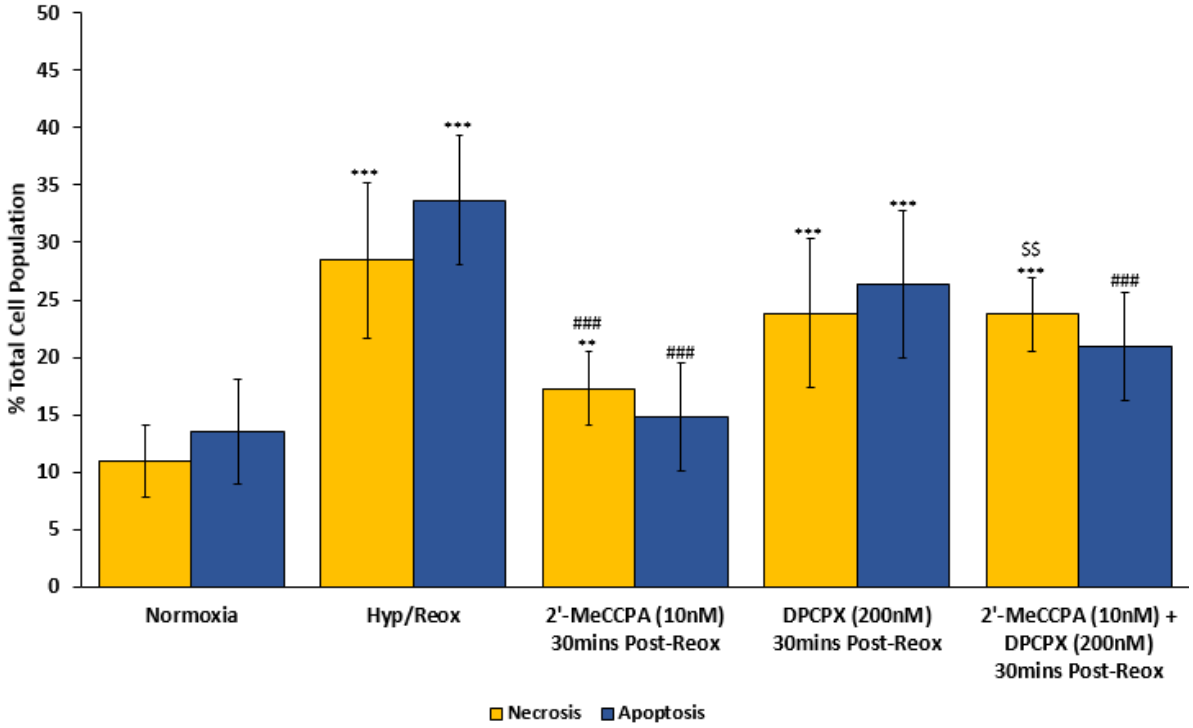


Figure 4. 15 The assessment of necrosis and apoptosis within isolated rat cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation. 2'-MeCCPA (10nM) was administered at 15 minutes post-reoxygenation in the presence and absence of A_1 adenosine receptor antagonist, DPCPX (200nM). Results were shown as Mean±SEM and were expressed as a percentage of 10 000 cells counted from 6 individual experiments. *** p<0.001 vs. Normoxia. ** p<0.01 vs. Normoxia. ### p<0.001 vs. Hyp/Reox. $$ p<0.01 vs. 2'-MeCCPA 30mins Post-Reox.

4.3.1.8 Profiling effects of co-administration of 2'-MeCCPA (10nM) with A_1 adenosine antagonist, DPCPX (200nM) at the onset of reoxygenation, 15 minutes post-reoxygenation and 30 minutes post-reoxygenation on cleaved caspase-3 activity within isolated rat cardiomyocytes when subjected to 1 hour of hypoxia and 3 hours of reoxygenation

Isolated adult rat cardiomyocytes were subjected to 1 hour of hypoxia and 3 hours of reoxygenation. It was found when 2'-MeCCPA (10nM) + DPCPX (200nM) was administered in conjunction together, there was a significant increase in cleaved-caspase 3 activity in comparison to when 2'-MeCCPA (10nM) was administered alone at the onset of reoxygenation (retrospectively, 238±20% vs. 181±35%, p<0.05) (Figure 4.16).

When 2'-MeCCPA + DPCPX was administered at 15 minutes post-reoxygenation, there was a significant increase in cleaved-caspase 3 activity when compared to 2'-MeCCPA being

administered alone at 15 minutes post-reoxygenation (retrospectively, 313±37% vs. 232±33%, p<0.05) (Figure 4.17). When DPCPX was administered alone, there was an increase in cleaved-caspase 3 activity when compared to 2'-MeCCPA being administered alone however this was not a significant change.

When 2'-MeCCPA + DPCPX was administered in conjunction at 30 minutes post-reperfusion, there was a slight increase in cleaved-caspase 3 activity when compared to 2'-MeCCPA being administered alone at 30 minutes post-reoxygenation however this was not a significant change (retrospectively, 283±31% vs. 263±68%, no significance p>0.05).

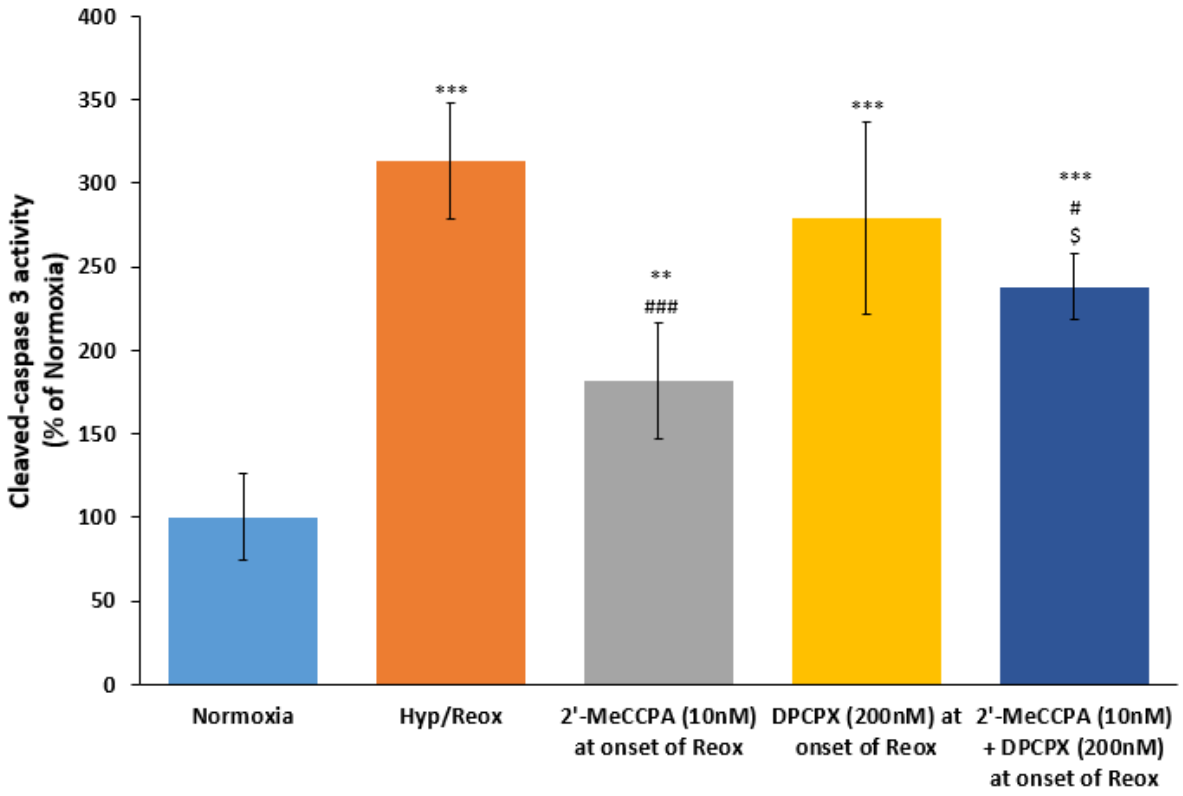


Figure 4. 16 Cleaved-caspase 3 activity in isolated rat cardiomyocytes subjected to 4 hours of reoxygenation (Normoxia) or 1 hour of hypoxia followed by 3 hours of reoxygenation (Hyp/Reox). 2'-MeCCPA (10nM) was administered in the presence and absence of A_1 adenosine receptor antagonist, DPCPX (200nM) at the onset of reoxygenation. Data presented as percentage of Normoxia±SEM n=6. *** p<0.001 vs. Normoxia. ** p<0.01 vs. Normoxia. ### p<0.001 vs. Hyp/Re. # p<0.05 vs. Hyp/Re. $ p<0.05 vs. 2'-MeCCPA at onset of Reox.

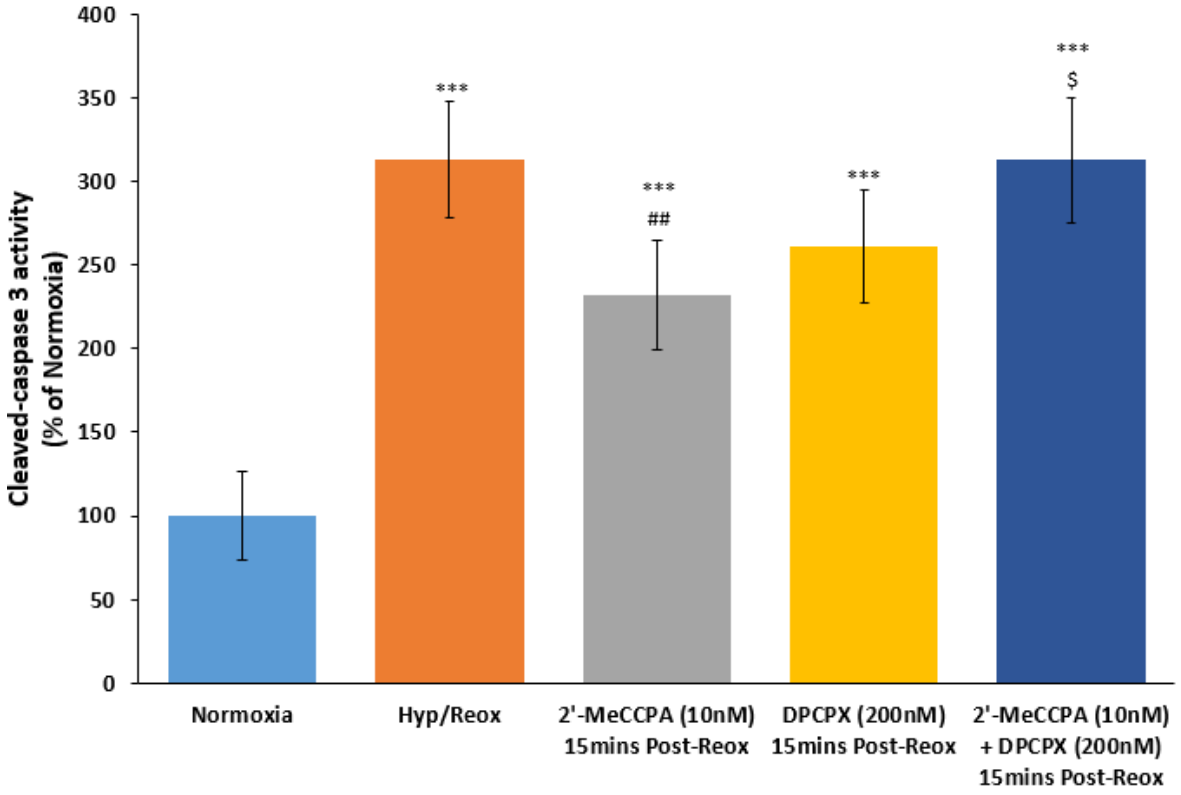


Figure 4. 17 Cleaved-caspase 3 activity in isolated rat cardiomyocytes subjected to 4 hours of reoxygenation (Normoxia) or 1 hour of hypoxia followed by 3 hours of reoxygenation (Hyp/Reox). 2'-MeCCPA (10nM) was administered in the presence and absence of A_1 adenosine receptor antagonist, DPCPX (200nM) at 15 minutes post-reoxygenation. Data presented as percentage of Normoxia±SEM n=6. *** p<0.001 vs. Normoxia. ## p<0.0 vs. Hyp/Reox. $$$ p<0.001 vs. 2'-MeCCPA 15mins Post-R.

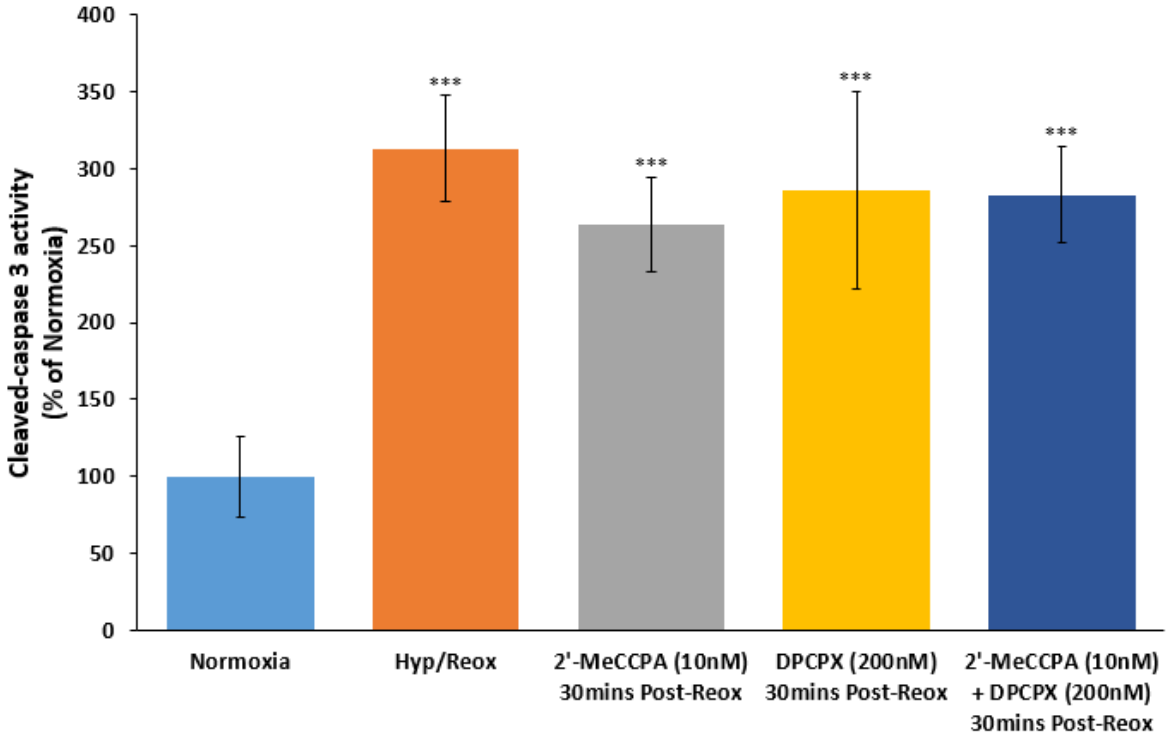


Figure 4. 18 Cleaved-caspase 3 activity in isolated rat cardiomyocytes subjected to 4 hours of reoxygenation (Normoxia) or 1 hour of hypoxia followed by 3 hours of reoxygenation (Hyp/Reox). 2'-MeCCPA (10nM) was administered in the presence and absence of A_1 adenosine receptor antagonist, DPCPX (200nM) at 30 minutes post-reoxygenation. Data presented as percentage of Normoxia±SEM n=6. *** $p<0.001$ vs. Normoxia.

4.3.1.9 Administration of A_1AR agonist 2'-MeCCPA (10nM) in the presence and absence of A_1AR antagonist DPCPX (200nM) at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion and the effects upon p-AKT (Ser473) phosphorylation in western blots

It has previously been shown that when 2'-MeCCPA (10nM) is administered at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion, there is a significant decrease in infarct size to risk ratio however when 2'-MeCCPA (10nM) is administered in the presence of the A_1AR antagonist DPCPX (200nM), there is a significant increase in infarct size to risk ratio at all different time points meaning protection was blocked in the presence of DPCPX (200nM).

In order to furthermore determine if the protection shown by the administration of 2'-MeCCPA (10nM) at the onset of reperfusion, 15 minutes and 30 minutes into the onset of reperfusion explicitly involved the PI3K-AKT pro-survival pathway, all heart tissue was treated with 2'-MeCCPA (10nM) in the presence and absence of DPCPX (200nM) either at the onset of reperfusion, 15 minutes post-reperfusion or 30 minutes post-reperfusion. Heart tissue was treated with drug intervention and then further assessed for p-AKT $_{(ser473)}$ protein phosphorylation which was normalised against the total AKT $_{(Ser473)}$ protein phosphorylation through western blot analysis.

The western blot analysis indicated that when 2'-MeCCPA (10nM) was administered at the onset of reperfusion, there was a significant upregulation in the levels of p-AKT $_{(Ser473)}$ when compared to the control group (284 ± 10% vs. 116 ± 14%, $p<0.001$). Furthermore the administration of 2'-MeCCPA (10nM) with DPCPX (200nM) significantly decreased the levels of phosphorylated AKT when compared to the 2'-MeCCPA (10nM) alone group (103 ± 15% vs. 284 ± 10%, $p<0.001$) (Figure 4.19).

Furthermore western blot analysis showed that the administration of 2'-MeCCPA (10nM) at 15 minutes post-reperfusion indicated a significant increase in levels of p-AKT$_{(Ser473)}$ when compared to the control group (267 ± 16% vs 163 ± 13%, $p<0.001$). It was also found that when 2'-MeCCPA (10nM) was co-administered with DPCPX (200nM), there was a significant downregulation in p-AKT levels in comparison to the 2'-MeCCPA alone at 15 minutes post-reperfusion group (99 ± 27% vs. 267 ± 16%, $p<0.001$) (Figure 4.20).

When 2'-MeCCPA (10nM) was administered alone at 30 minutes post-reperfusion, the western blot analysis determined that there was a significant upregulation in p-AKT $_{(Ser473)}$ levels compared to the control group (206 ± 18% vs. 124 ± 11%, $p<0.001$). When 2'-MeCCPA (10nM) was administered alongside DPCPX (200nM) at 30 minutes post-reperfusion, there was a significant downregulation in p-AKT phosphorylation compared to the 2'-MeCCPA alone at 30 minutes post-reperfusion group (120 ± 21% vs. 206 ± 18%, $p<0.01$) (Figure 4.21).

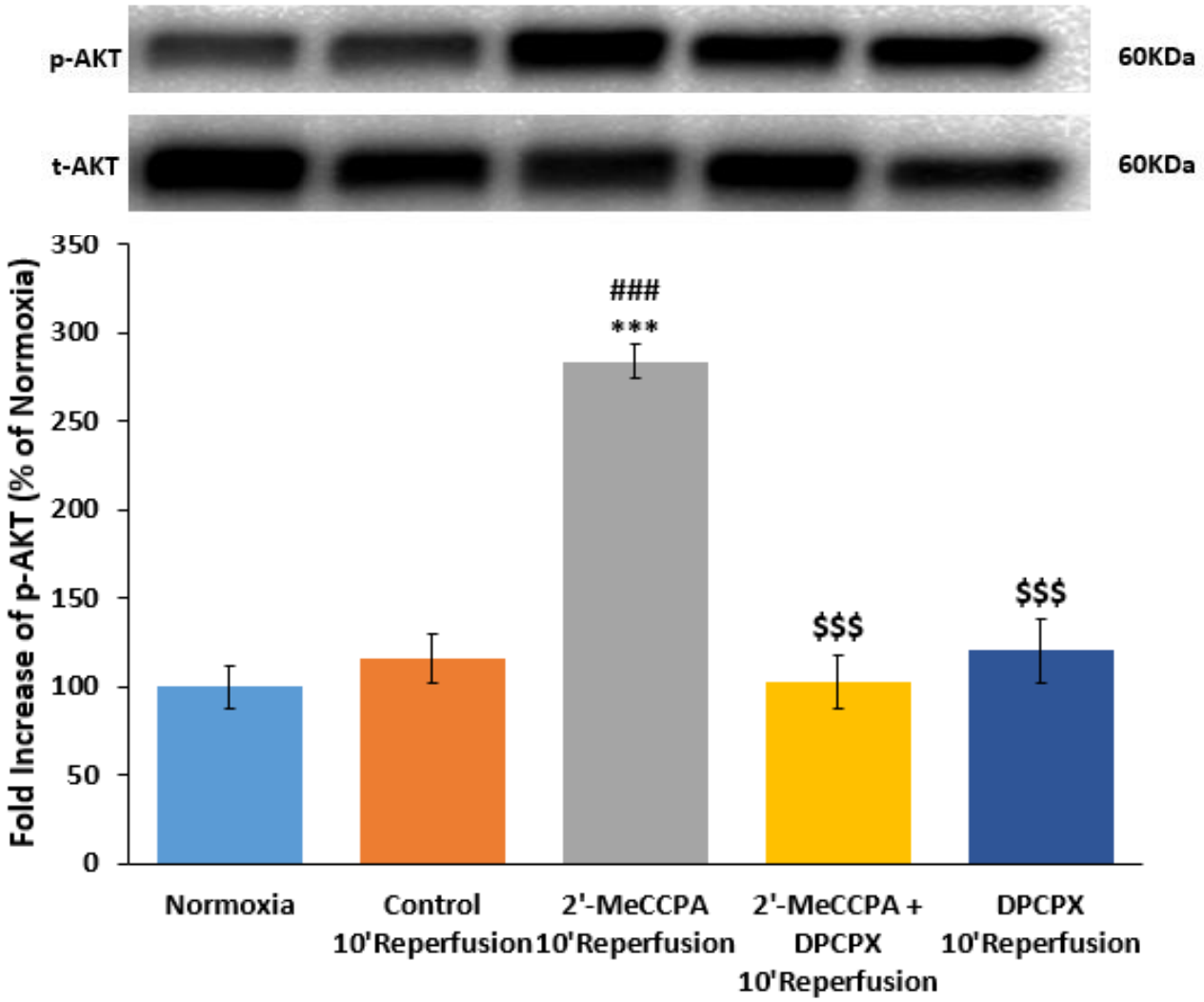


Figure 4. 19 Western blot analysis showing the effects of A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of A_1AR antagonist DPCPX (200nM) at the onset of reperfusion. Reperfusion time went on for 10 minutes after the onset and heart tissue was then stored. Results were shown as Mean ± SEM of 4 experiments. ***p<0.001 vs. Normoxia. ###p<0.001 vs. Control. $$$p<0.001 vs. 2'-MeCCPA.

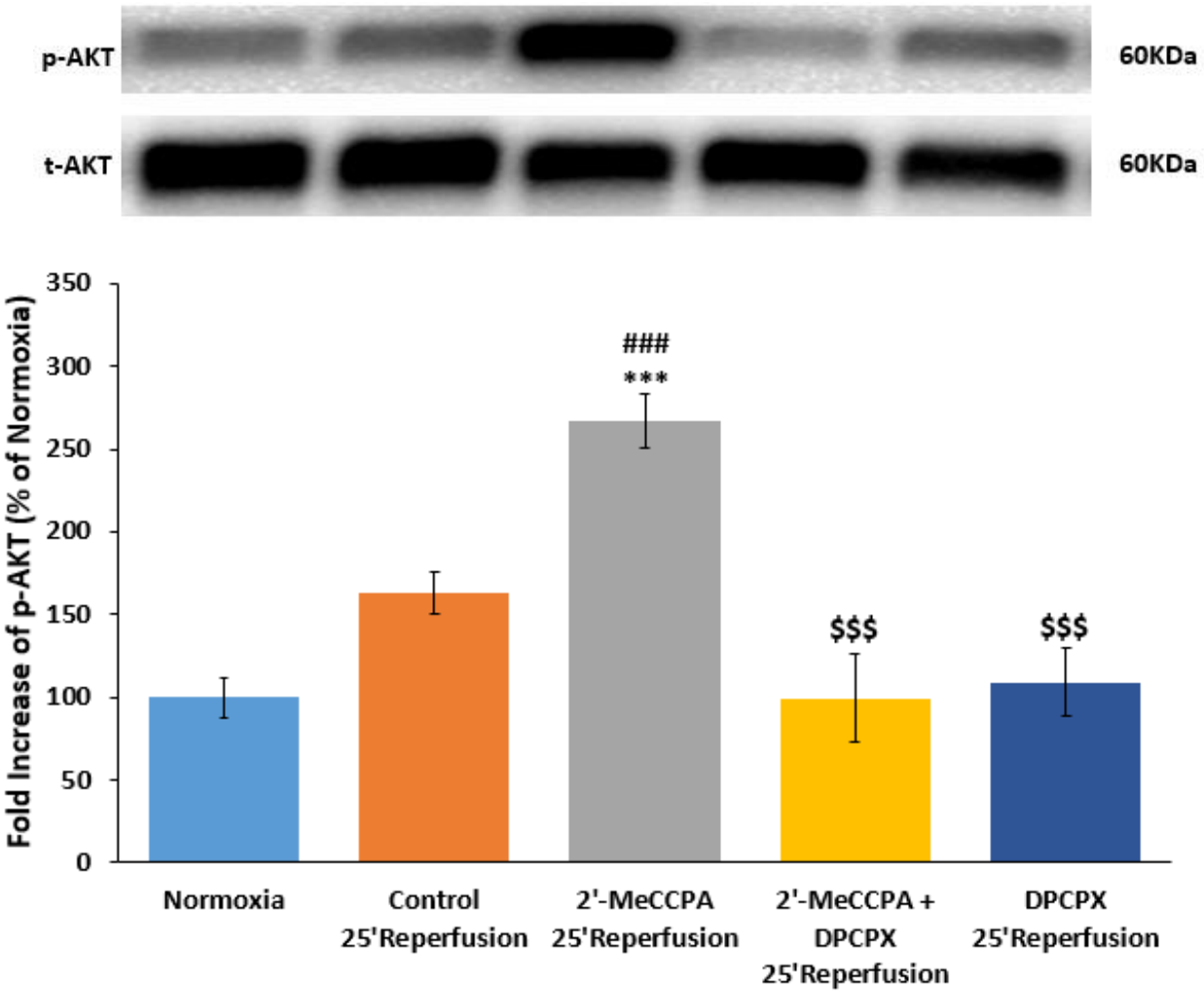


Figure 4. 20 Western blot analysis showing the effects of A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of A_1AR antagonist DPCPX (200nM) when administered 15 minutes post-reperfusion. Reperfusion time went on for a further 10 minutes after drug administration making a total 25 minutes reperfusion period and then heart tissue was stored. Results were shown as Mean ± SEM of 4 experiments. ***p<0.001 vs. Normoxia. ###p<0.001 vs. Control. $$$p<0.001 vs. 2'-MeCCPA.

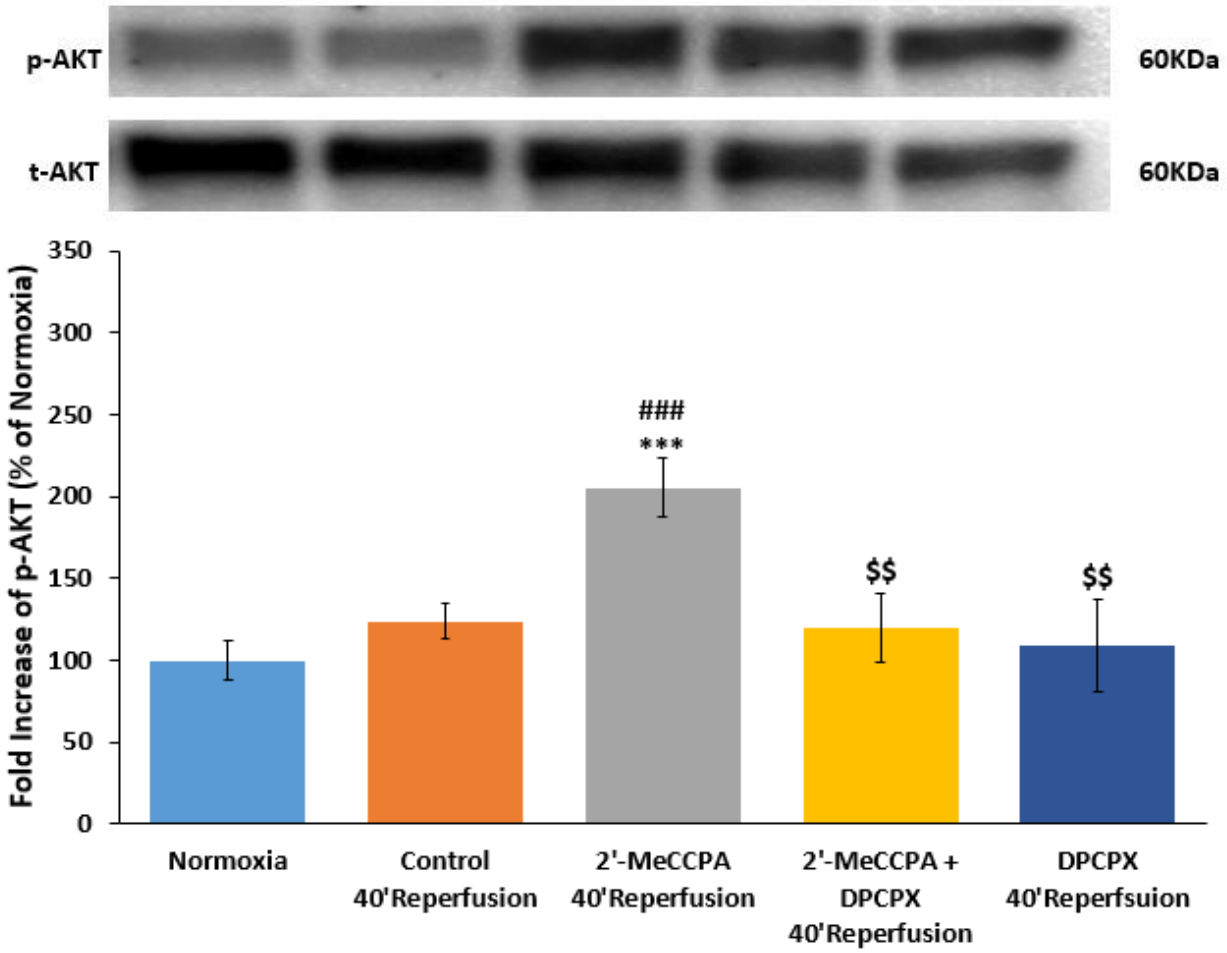


Figure 4. 21 Western blot analysis showing the effects of A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of A_1AR antagonist DPCPX (200nM) when administered 30 minutes post-reperfusion. Reperfusion time went on for a further 10 minutes after drug administration making a total 40 minutes reperfusion period and then heart tissue was stored. Results were shown as Mean ± SEM of 4 experiments. ***p<0.001 vs. Normoxia. ###p<0.001 vs. Control. $$p<0.01 vs. 2'-MeCCPA.

4.3.2 The effects of co-administration of 2'-MeCCPA (10nM) with unselective adenosine antagonist, 8-SPT (1µM) at various time-points within reperfusion in myocardial ischaemia reperfusion injury.

4.3.2.1 The effects of co-administration of 2'-MeCCPA (10nM) + 8-SPT (1µM) on infarct size to risk ratio within isolated hearts subjected to ischaemia reperfusion injury at the onset of reperfusion.

When 2'-MeCCPA (10nM) was administered alone at reperfusion, there was a significant decrease in infarct size to risk ratio (%) compared to the IR control (28 ± 4% vs. 55 ± 6%, $p<0.001$) (Figure 4.22). When unselective adenosine antagonist 8-SPT (1µM) was administered alongside 2'-MeCCPA (10nM) at the onset of reperfusion, no significant change was observed in comparison to when 2'-MeCCPA (10nM) was administered alone at reperfusion (39 ± 3% 2'-MeCCPA + 8-SPT vs. 28 ± 5% 2'-MeCCPA, $p>0.05$) (Figure 4.22). When 2'-MeCCPA (10nM) + 8-SPT (1µM) was administered together, this showed a significant decrease in infarct size to risk ratio in comparison to the IR control (39 ± 3% 2'-MeCCPA+8-SPT vs. 55 ± 6% IR control, $p<0.05$) (Figure 4.22).

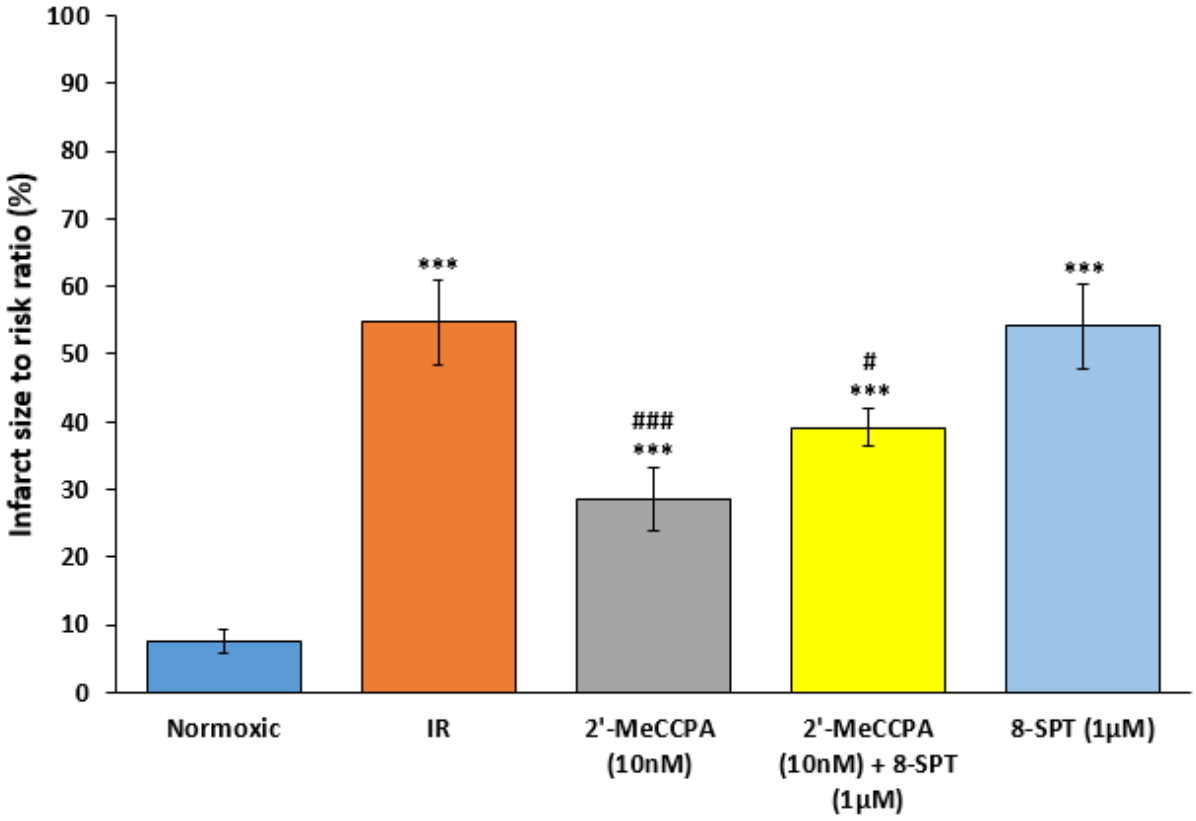


Figure 4. 22 Infarct size to risk ratio (%) within isolated perfused rats' hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Hearts were subjected to A_1AR agonist 2'-MeCCPA (10nM) administered at the onset of reperfusion in the presence and absence of unselective adenosine antagonist 8-SPT (1µM). Data presented as Mean±SEM. n=6-8. *** p<0.001 vs. normoxic, ### p<0.001 vs. IR and # p<0.05 vs. IR.

4.3.2.2 The effects of co-administration of 2'-MeCCPA (10nM) + 8-SPT (1µM) on infarct size to risk ratio within isolated hearts subjected to ischaemia reperfusion injury at 15 minutes post-reperfusion.

2'-MeCCPA (10nM) administered alone at 15 minutes post-reperfusion showed a significant decrease in infarct size to risk ratio (%) compared to the IR control (30 ± 8% 2'-MeCCPA 15mins Post-R vs 55 ± 6% IR control, p<0.001) (Figure 4.23). When 2'-MeCCPA (10nM) was administered alongside 8-SPT (1µM) at 15 minutes post-reperfusion, there was a significant increase in infarct size to risk ratio (%) in comparison to when 2'-MeCCPA (10nM) was administered alone at 15 minutes post-reperfusion (45 ± 3% 2'-MeCCPA + 8-SPT 15mins Post-R vs. 30 ± 8% 2'-MeCCPA 15mins Post-R, p<0.05) (Figure 4.23). When 8-SPT (1µM) was administered alone at 15 minutes post-reperfusion, there was a significant increase in infarct

size to risk ratio (%) compared to when 2'-MeCCPA (10nM) was administered alone at 15 minutes post-reperfusion (56 ± 5% 8-SPT 15mins Post-R vs. 30 ± 8% 2'-MeCCPA 15mins Post-R, p<0.05) (Figure 4.23).

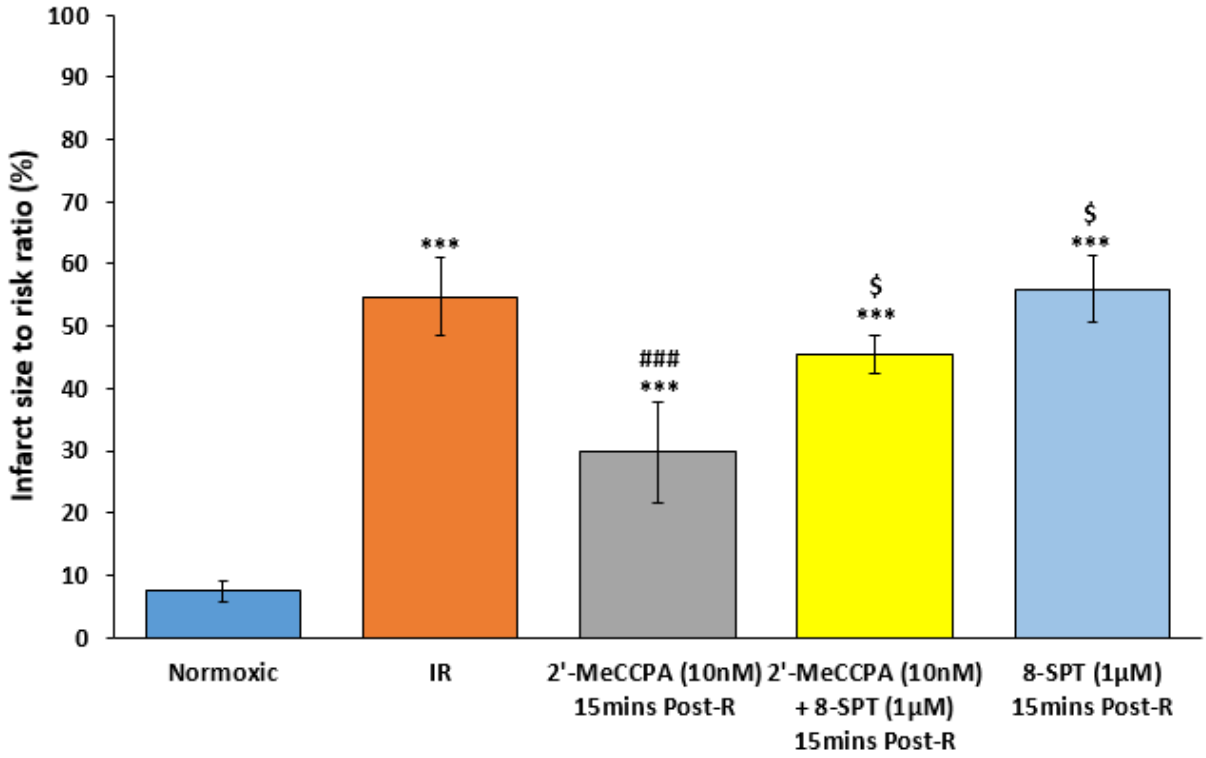


Figure 4. 23 Infarct size to risk ratio (%) within isolated perfused rats' hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Hearts were subjected to A_1AR agonist 2'-MeCCPA (10nM) administered at 15 minutes post-reperfusion in the presence and absence of unselective adenosine antagonist 8-SPT (1µM). Data presented as Mean±SEM. n=6-8. *** p<0.001 vs. normoxic, ### p<0.001 vs. IR and $ p<0.05 vs. 2'-MeCCPA 15mins Post-R.

4.3.2.3 Effects of co-administration of 2'-MeCCPA (10nM) + 8-SPT (1µM) on infarct size to risk ratio within isolated hearts subjected to ischaemia reperfusion injury at 30 minutes post-reperfusion.

When 2'-MeCCPA (10nM) was administered alone at 30 minutes post-reperfusion, there was a significant decrease in infarct size to risk ratio (%) compared to the IR control (35 ± 6% 2'-MeCCPA 30mins Post-R vs. 55 ± 6% IR control, p<0.001) (Figure 4.24). When 2'-MeCCPA (10nM) and 8-SPT (1µM) was administered in conjunction, there was a significant increase in infarct size to risk ratio compared to when 2'-MeCCPA (10nM) was administered alone at 30 minutes post-reperfusion (48 ± 4 2'-MeCCPA + 8-SPT 30mins Post-R vs. 2'-MeCCPA 30mins

Post-R, p<0.05) (Figure 4.24). When 8-SPT (1µM) was administered alone at 30 minutes post-reperfusion, there was a significant increase in infarct size to risk ratio compared to when 2'-MeCCPA (10nM) was administered alone at 30 minutes post-reperfusion (53 ± 5% 8-SPT 30mins Post-R vs. 35 ± 6% 2'-MeCCPA 30mins Post-R, p<0.05) (Figure 4.24).

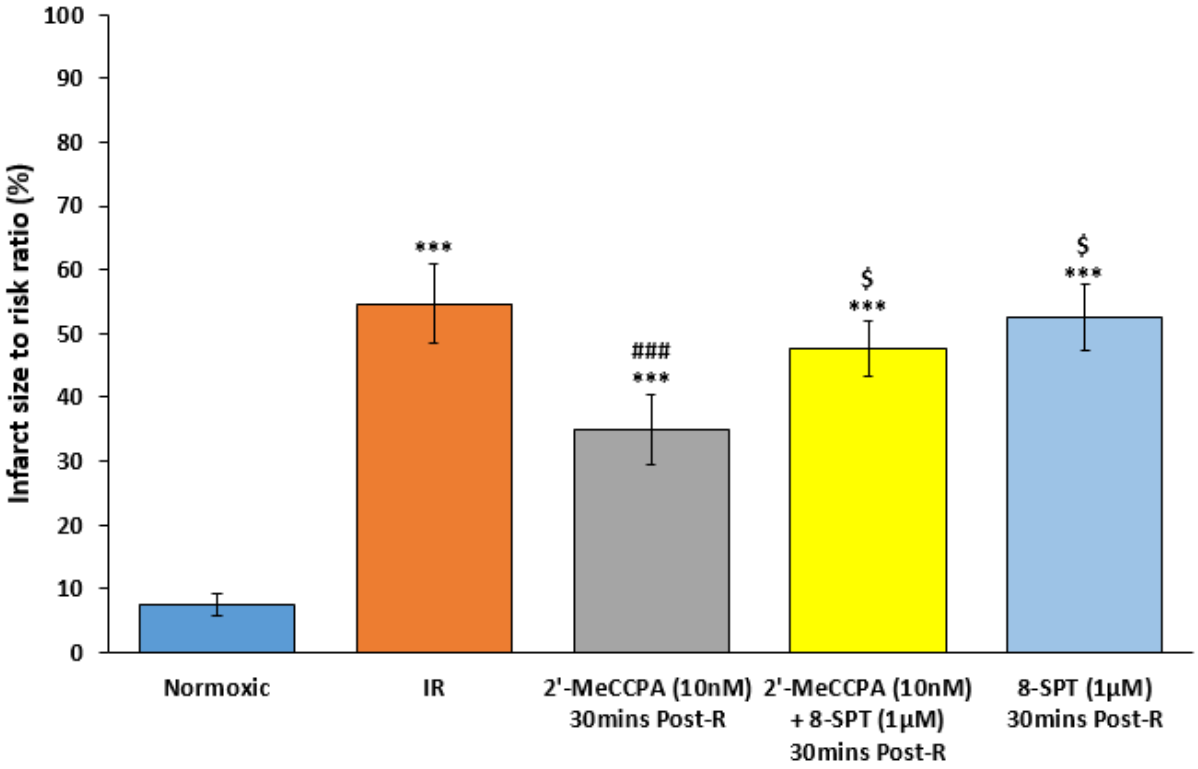


Figure 4. 24 Infarct size to risk ratio (%) within isolated perfused rats' hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Hearts were subjected to A_1AR agonist 2'-MeCCPA (10nM) administered at 30 minutes post-reperfusion in the presence and absence of unselective adenosine antagonist 8-SPT (1µM). Data presented as Mean±SEM. n=6-8. *** p<0.001 vs. normoxic, ### p<0.001 vs. IR and $ p<0.05 vs. 2'-MeCCPA 30mins Post-R.

4.3.2.4 Profiling effects of co-administration of 2'-MeCCPA (10nM) alongside A_1 adenosine antagonist, DPCPX (200nM) at the onset of reoxygenation, 15 minutes post-reoxygenation and 30 minutes post-reoxygenation in adult rat cardiomyocytes subjected to hypoxia-reoxygenation on apoptosis and necrosis

4.3.2.4a Effects of administration of A_1AR agonist 2'-MeCCPA (10nM) + 8-SPT (1µM) on apoptosis and necrosis at the onset of reperfusion

When 2'-MeCCPA (10nM) was administered alongside unselective adenosine antagonist, 8-SPT (1µM), there was a significant increase in apoptosis (27 ± 4% 2'-MeCCPA + 8-SPT vs. 17 ± 4% 2'-MeCCPA, $p<0.01$) and necrosis (23 ± 3% 2'-MeCCPA + 8-SPT vs. 13 ± 2% 2'-MeCCPA, $p<0.05$) compared to when 2'-MeCCPA (10nM) was administered alone (Figure 4.25).

No significant differences in apoptosis and necrosis were detected when 8-SPT (1µM) was administered alone at the onset of reperfusion ($p>0.05$).

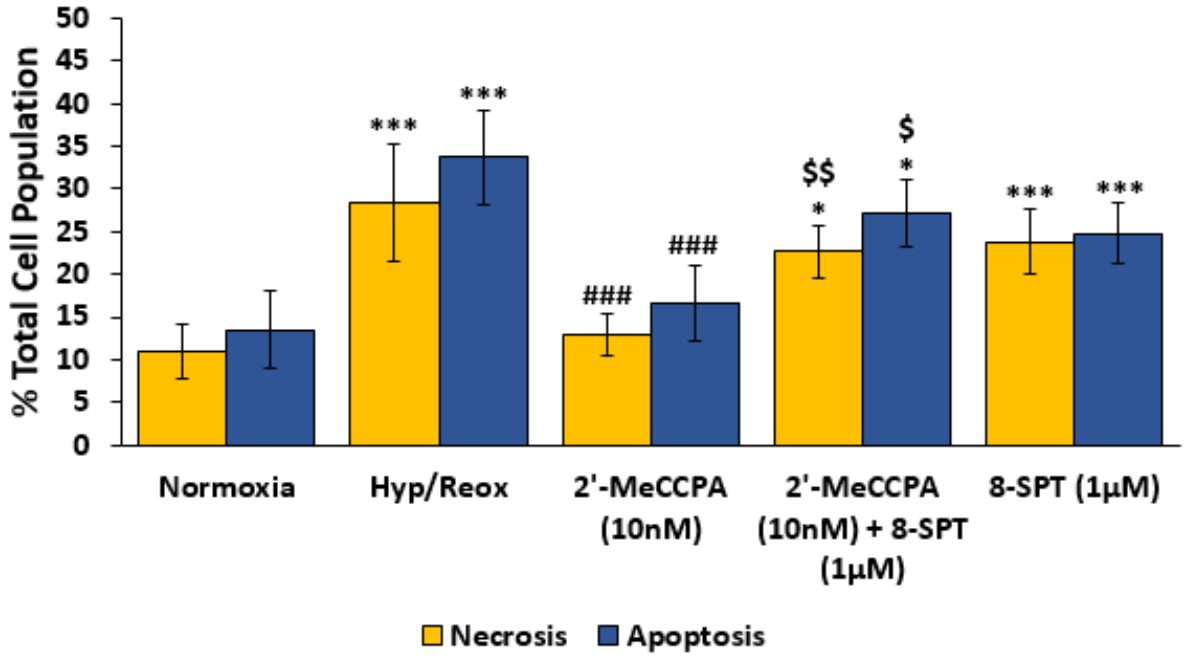


Figure 4. 25 Assessment of necrosis and apoptosis within isolated rat cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation. 2'-MeCCPA (10nM) was administered at the onset of reoxygenation in the presence and absence of unselective adenosine antagonist (1µM). Results were shown as Mean±SEM and were expressed as a percentage of 10 000 cells counted from 6 individual experiments. *** $p<0.001$ vs. Normoxia.** $p<0.01$ vs. Normoxia. ### $p<0.001$ vs. Hyp/Reox. # $p<0.05$ vs. Hyp/Reox.

4.3.2.4b Effects of administration of A_1AR agonist 2'-MeCCPA (10nM) + 8-SPT (1µM) on apoptosis and necrosis at 15 minutes post-reoxygenation

When 2'-MeCCPA (10nM) was administered at 15 minutes post-reoxygenation, a significant decrease in apoptosis was observed compared to the Hyp/Reox control group (14 ± 5% 2'-MeCCPA 15mins Post-R vs. 34 ± 6% Hyp/Reox control, $p<0.001$) (Figure 4.26); a significant decrease in necrosis was also observed when 2'-MeCCPA (10nM) was administered alone at 15 minutes post-reoxygenation (16 ± 4% 2'-MeCCPA 15mins Post-R vs. 28 ± 7%, $p<0.001$) (Figure 4.26).

An increase in necrosis was detected when 2'-MeCCPA (10nM) and 8-SPT (1µM) were administered at 15 minutes post-reoxygenation compared to when 2'-MeCCPA (10nM) was administered alone at 15 minutes post-reoxygenation however this was not a significant change ($p>0.05$) (Figure 4.26). An increase in apoptosis was detected when 2'-MeCCPA (10nM) and 8-SPT (1µM) were administered at 15 minutes post-reoxygenation compared to when 2'-MeCCPA (10nM) was administered alone at 15 minutes post-reoxygenation, however this was also not a significant change ($p>0.05$) (Figure 4.26).

No significant difference in apoptosis and necrosis was detected when 8-SPT (1µM) was administered at 15 minutes post-reoxygenation compared to the Hyp/Reox control group ($p>0.05$) (Figure 4.26).

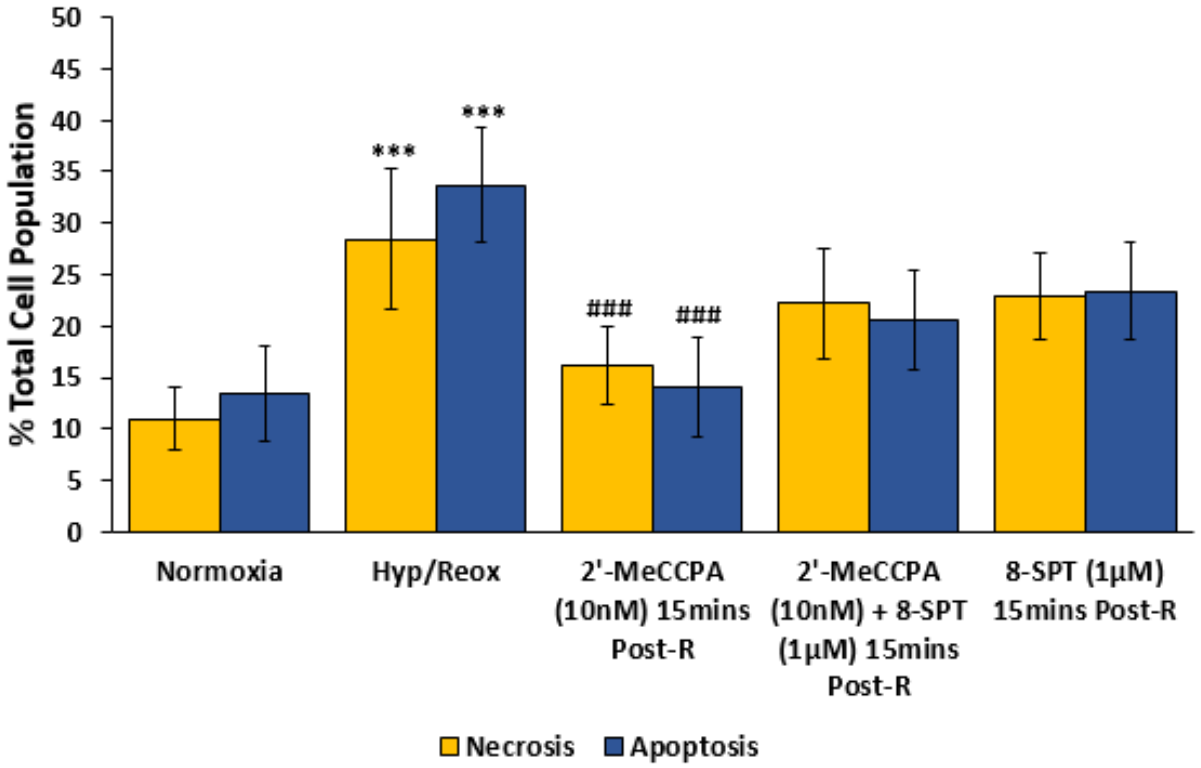


Figure 4. 26 Assessment of necrosis and apoptosis within isolated rat cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation. 2'-MeCCPA (10nM) was administered at 15 minutes post-reoxygenation in the presence and absence of unselective adenosine antagonist (1µM). Results were shown as Mean±SEM and were expressed as a percentage of 10 000 cells counted from 6 individual experiments. *** p<0.001 vs. Normoxia.** p<0.01 vs. Normoxia. ### p<0.001 vs. Hyp/Reox.

4.3.2.4c Effects of administration of A_1AR agonist 2'-MeCCPA (10nM) alongside unselective adenosine antagonist 8-SPT (1µM) on apoptosis and necrosis at 30 minutes post-reoxygenation

When 2'-MeCCPA (10nM) was administered at 30 minutes post-reoxygenation there was a significant decrease in apoptosis compared to the Hyp/Reox control (15 ± 5% 2'-MeCCPA 15mins Post-R vs. 34 ± 6% Hyp/Reox, p<0.001) (Figure 4.27). When 2'-MeCCPA (10nM) was administered at 30 minutes post-reoxygenation, there was a significant decrease in necrosis compared to the Hyp/Reox control group (17 ± 3% 2'-MeCCPA 15mins Post-R vs. 28 ± 7 Hyp/Reox, p<0.001) (Figure 4.27).

No significant change was detected in apoptosis and necrosis when 2'-MeCCPA (10nM) was administered alongside 8-SPT (1µM) at 30 minutes post-reoxygenation compared to when

2’-MeCCPA (10nM) was administered alone at 30 minutes post-reoxygenation (p>0.05 (Figure 4.27).

Furthermore, no significant difference was detected in apoptosis and necrosis was when 8-SPT (1µM) was administered at 30 minutes post-reoxygenation compared to the Hyp/Reox control group (p>0.05) (Figure 4.27).

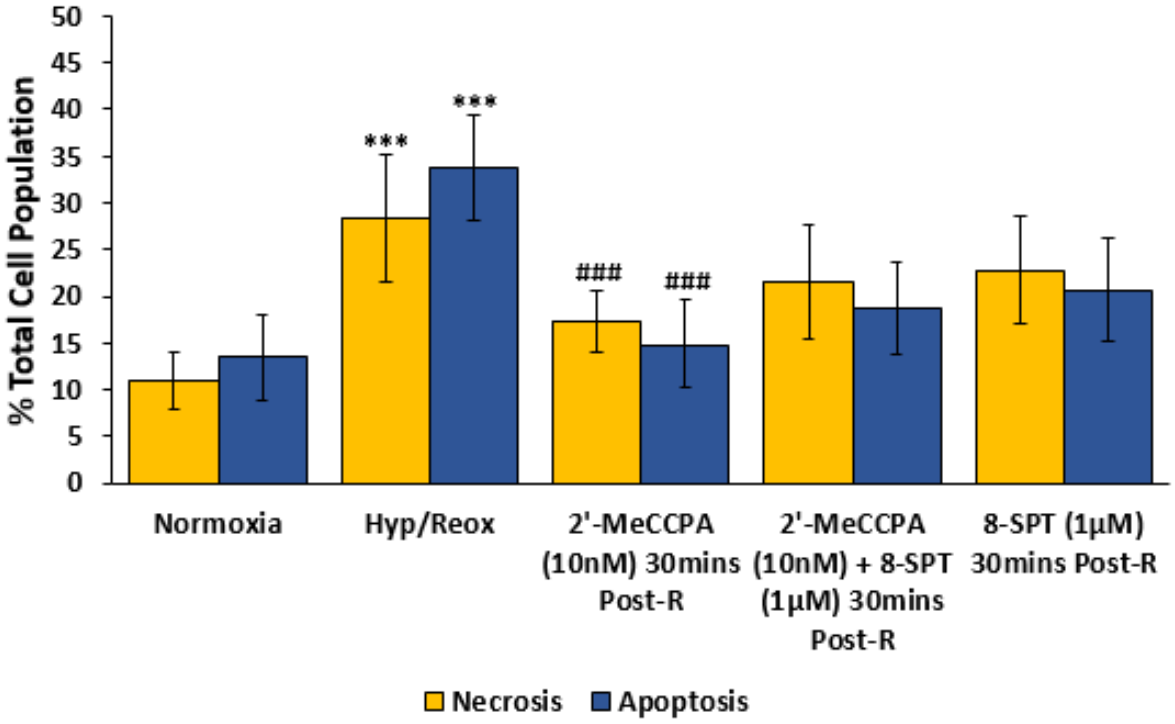


Figure 4. 27 Assessment of necrosis and apoptosis within isolated rat cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation. 2’-MeCCPA (10nM) was administered at 30 minutes post-reoxygenation in the presence and absence of unselective adenosine antagonist (1µM). Results were shown as Mean±SEM and were expressed as a percentage of 10 000 cells counted from 6 individual experiments. *** p<0.001 vs. Normoxia. ### p<0.001 vs. Hyp/Reox.

4.3.2.5 Profiling effects of co-administration of 2'-MeCCPA (10nM) with A_1 adenosine antagonist, DPCPX (200nM) at the onset of reoxygenation, 15 minutes post-reoxygenation and 30 minutes post-reoxygenation on cleaved caspase-3 activity within isolated rat cardiomyocytes when subjected to 1 hour of hypoxia and 3 hours of reoxygenation

4.3.2.5a Effects of administration of A_1AR agonist 2'-MeCCPA (10nM) + 8-SPT (1µM) on cleaved capase-3 activity at the onset of reperfusion

When 2'-MeCCPA (10nM) was administered in conjunction with unselective adenosine antagonist 8-SPT (1µM), there was an increase in cleaved caspase-3 activity compared to when 2'-MeCCPA (10nM) was administered alone (287 ± 22% 2'-MeCCPA + 8-SPT vs. 181 ± 35% 2'-MeCCPA, $p<0.01$) (Figure 4.28). When 8-SPT (1µM) was administered alone at the onset of reoxygenation, there was no significant difference in comparison to the Hyp/Reox control group.

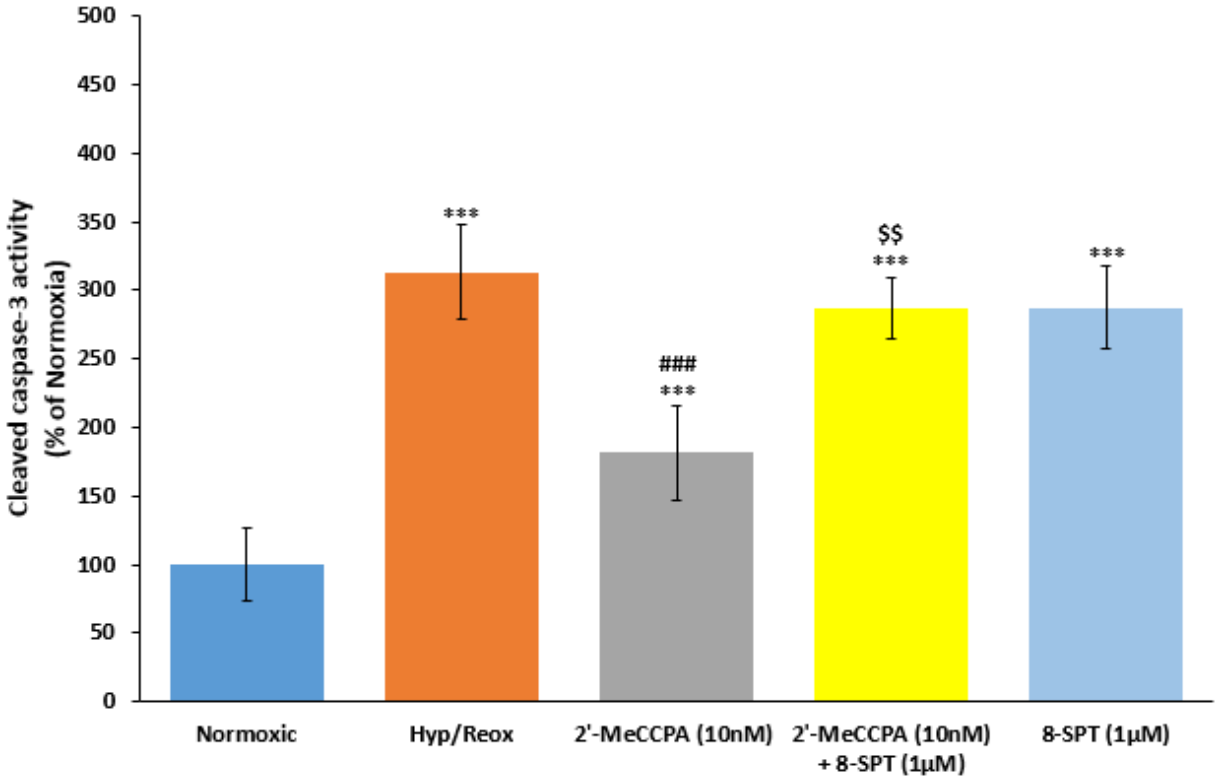


Figure 4. 28 Cleaved-caspase 3 activity in isolated rat cardiomyocytes subjected to 4 hours of reoxygenation (Normoxia) or 1 hour of hypoxia followed by 3 hours of reoxygenation (Hyp/Reox). 2'-MeCCPA (10nM) was administered in the presence and absence of unselective adenosine receptor antagonist, 8-SPT (1µM) at the onset of reoxygenation. Data presented as percentage of Normoxia±SEM n=6. *** $p<0.001$ vs. Normoxia. ### $p<0.001$ vs. Hyp/Re. $$ $p<0.01$ vs. 2'-MeCCPA.

4.3.2.5b Effects of administration of A_1AR agonist 2'-MeCCPA (10nM) + 8-SPT (1µM) on cleaved capase-3 activity at 15 minutes post-reoxygenation

When 2'-MeCCPA (10nM) was administered at 15 minutes post-reoxygenation, there was a significant decrease in cleaved caspase-3 activity when compared to the Hyp/Reox control group (232 ± 39% 2'-MeCCPA 15mins Post-R vs. 313 ± 35% Hyp/Reox, $p<0.05$) (Figure 4.29).

When 2'-MeCCPA (10nM) and 8-SPT (1µM) were administered together at 15 minutes post-reoxygenation, there was a significant increase in cleaved caspase-3 activity compared to when 2'-MeCCPA (10nM) was administered alone at 15 minutes post-reoxygenation (334 ± 39% 2'-MeCCPA + 8-SPT 15mins Post-R vs. 232 ± 39% 2'-MeCCPA 15mins Post-R, $p<0.05$). (Figure 4.29).

No significant differences were detected when 8-SPT (1µM) was administered alone at 15 minutes post-reoxygenation (p>0.05) (Figure 4.29).

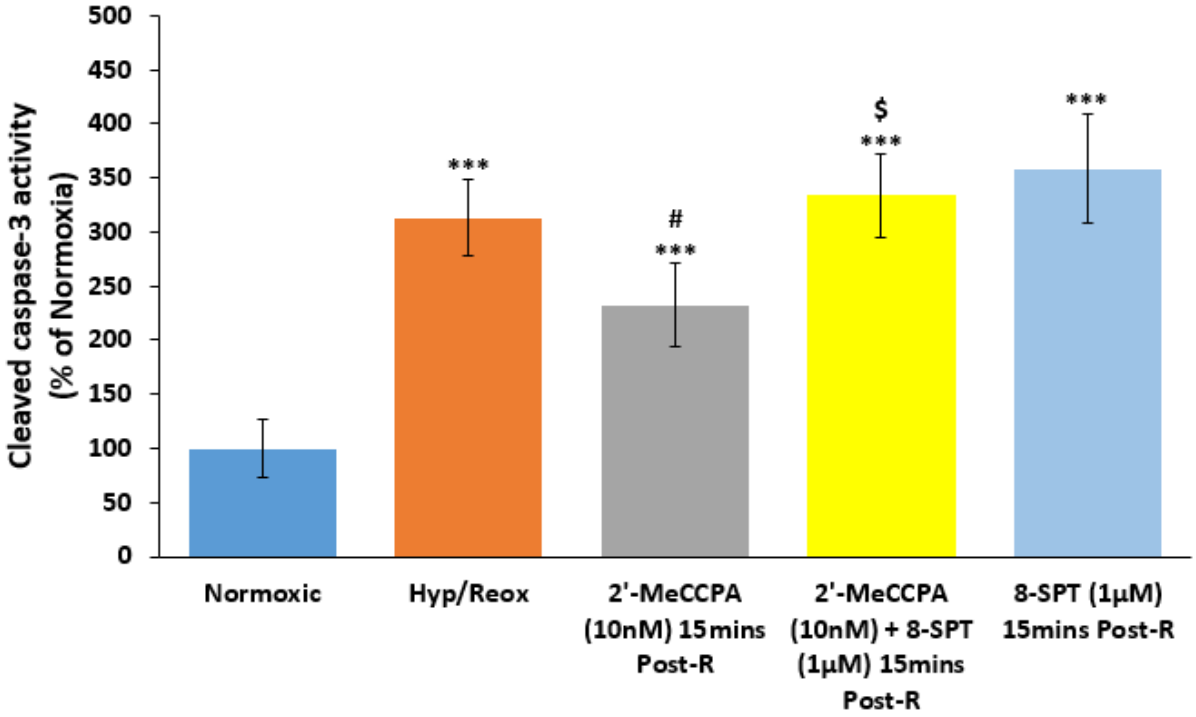


Figure 4. 29 Cleaved-caspase 3 activity in isolated rat cardiomyocytes subjected to 4 hours of reoxygenation (Normoxia) or 1 hour of hypoxia followed by 3 hours of reoxygenation (Hyp/Reox). 2'-MeCCPA (10nM) was administered in the presence and absence of unselective adenosine receptor antagonist, 8-SPT (1µM) at 15 minutes post-reoxygenation. Data presented as percentage of Normoxia±SEM n=6. *** p<0.001 vs. Normoxia. # p<0.05 vs. Hyp/Re. $ p<0.05 vs. 2'-MeCCPA.

4.3.2.5c Effects of administration of A_1AR agonist 2'-MeCCPA (10nM) + 8-SPT (1µM) on cleaved capase-3 activity at 30 minutes post-reoxygenation

When 2'-MeCCPA (10nM) was administered at 30 minutes post-reoxygenation, there was a decrease in cleaved caspase-3 activity compared to the Hyp/Reox control group however this was not a significant decrease (p>0.05) (Figure 4.30).

When 2'-MeCCPA (10nM) was administered in conjunction with 8-SPT (1µM) 30 minutes post-reoxygenation, an increase in cleaved caspase-3 activity was observed compared to when 2'-

MeCCPA (10nM) was administered alone at 30 minutes post-reoxygenation however this was no a significant increase ($p>0.05$) (Figure 4.30).

No significant differences were observed when 8-SPT (1µM) was administered alone at 30 minutes post-reoxygenation in comparison to the Hyp/Reox control group ($p>0.05$) (Figure 4.30).

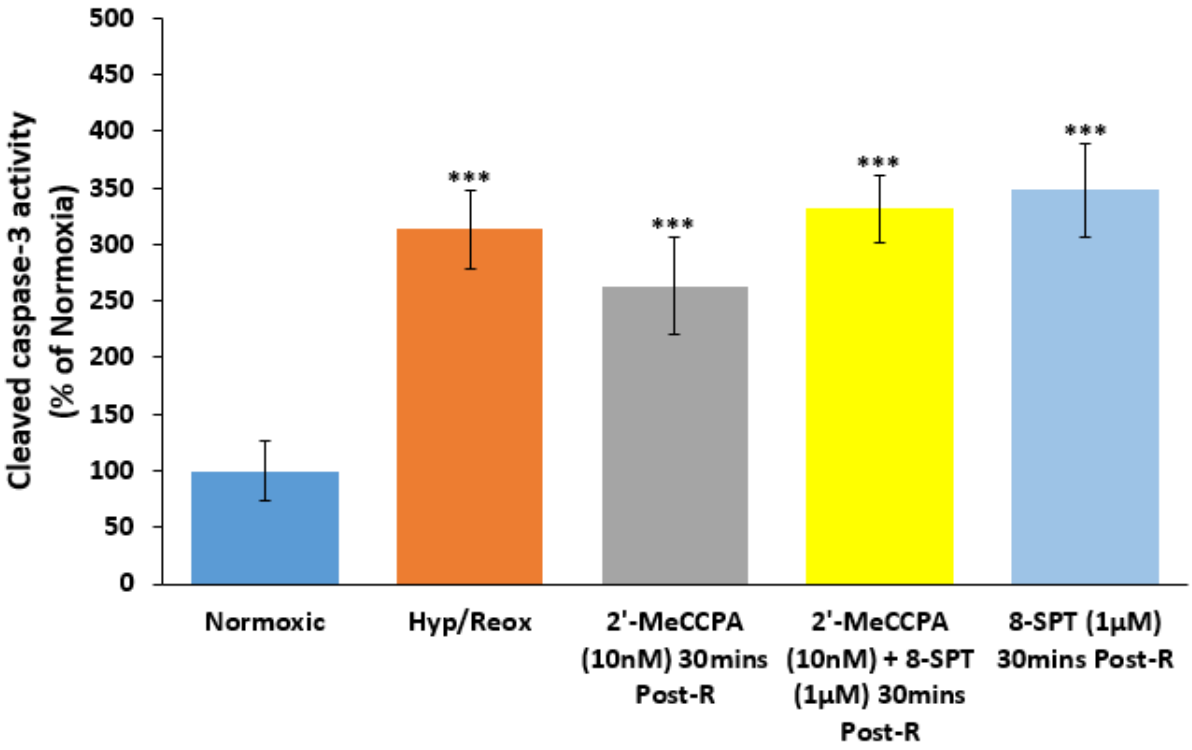


Figure 4. 30 Cleaved-caspase 3 activity in isolated rat cardiomyocytes subjected to 4 hours of reoxygenation (Normoxia) or 1 hour of hypoxia followed by 3 hours of reoxygenation (Hyp/Reox). 2'-MeCCPA (10nM) was administered in the presence and absence of unselective adenosine receptor antagonist, 8-SPT (1µM) at 30 minutes post-reoxygenation. Data presented as percentage of Normoxia±SEM n=6. *** $p<0.001$ vs. Normoxia. ### $p<0.001$ vs. Hyp/Re. $$ $p<0.01$ vs. 2'-MeCCPA.

4.4 Discussion

There have been numerous articles that have explored adenosine receptors as potential therapeutic targets (Jacobson and Gao 2006; Fredholm 2010; Chen et al. 2013; Covinhes et al. 2020; Gaundry et al. 2020). Research over the past 20 years has allowed for medicinal chemistry to generate various agonists and antagonists with affinity to selective adenosine receptor subtypes (Chen et al. 2013), however to date an effective adenosine related therapy has failed to translate to clinical settings.

Our findings within this chapter have explored the A_1 adenosine receptor subtype and its activation with A_1AR agonist 2'-MeCCPA. This chapter has also explored the effects of A_1AR antagonist DPCPX when administered alongside 2'-MeCCPA. The protection observed when 2'-MeCCPA (10nM) was administered alone at the onset of reperfusion was abrogated when 2'-MeCCPA (10nM) and DPCPX (200nM) were administered alongside each other at the onset of reperfusion. This can be supported by previous research conducted into the A_1 adenosine receptor where DPCPX has been shown to reverse any protective effects, for example a study by Louttit et al. (1999) researched into a selective A_1 adenosine agonist, GR79236 (10nM/kg) in an anaesthetised pig/porcine model of myocardial ischaemia-reperfusion injury. GR79236 was seen to show cardioprotective effects by reducing infarct size and when DPCPX was administered alongside it, all cardioprotective effects were reversed (Louttit et al. 1999). This is also very similar to our findings where 2'-MeCCPA successfully limited infarct size in an isolated rat heart model of myocardial ischaemia-reperfusion injury however when DPCPX was administered alongside at the onset of reperfusion, there was a significant increase in infarct size which therefore reversed cardioprotective effects. The findings from this study were also supported in an isolated mouse heart models of ischaemia-reperfusion (Urmaliya et al. 2010). This therefore implies that the cardioprotection observed is due to specific activation of the A_1 adenosine receptor as this protection is abrogated upon administration of A_1 adenosine receptor antagonist.

Our study further investigated into delayed post-reperfusion activation of the A_1 adenosine receptor at 15 minutes and 30 minutes post-reperfusion. Currently there is limited research that explores post-reperfusion activation using an A_1 adenosine receptor agonist however the study by Lubitz et al. (2001) successfully used the method of post-reperfusion activation of

the A_3 adenosine receptor using A_3 adenosine agonist IB-MECA which decrease infarct size following focal brain ischaemia. The methods of post-reperfusion activation of the A_3 adenosine receptor subtype draws direct links with our study in which post-reperfusion activation at 15 and 30 minutes of the A_1 adenosine receptor also successfully limited infarct size. Furthermore this study also administered the A_1 adenosine antagonist alongside 2'-MeCCPA at the two different delayed activation time points of 15 minutes and 30 minutes post-reperfusion and when DPCPX was administered, there was a significant increase in infarct size and abrogation in 2'-MeCCPA mediated protection. This therefore implies that the A_1 adenosine receptor subtype itself can provide cardioprotection. Later in the study the intracellular signalling pathways involved in the A_1 adenosine receptor mediated cardioprotection is explored using western blot analysis and the assessment of cell death and cleaved caspase-3 activity.

Alongside a decrease in infarct size in isolated rat hearts when 2'-MeCCPA (10nM) was administered alone at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion; and this protection abrogated in the presence of DPCPX (200nM). Cell death (apoptosis and necrosis) and cleaved caspase-3 activity was assessed. Overall when 2'-MeCCPA (10nM) was administered to isolated rat cardiomyocytes, there was a significant decrease in cellular apoptosis and necrosis however when DPCPX (200nM) was administered alongside, the level of cell death increased which in turn also increased the levels of cleaved caspase-3 at the points where 2'-MeCCPA and DPCPX was administered at the onset of reperfusion and also at 15 minutes post-reperfusion. When both 2'-MeCCPA and DPCPX were administered in conjunction at 30 minutes post-reperfusion, there was limited change in apoptosis and cleaved caspase-3 activity when compared to 2'-MeCCPA being administered alone at 30 minutes post-reperfusion. This could have occurred due to the 30 minutes time-point being a long duration after the onset of reperfusion to have 2'-MeCCPA activating the A_1 receptor. Hausenloy and Yellon (2003) stated that most protection occurs in the early stages of reperfusion and therefore this could support the findings of this current study in stating that the 30 minutes time point could just be too long after the commencement of reperfusion in order to ensure protection. Apoptosis could be occurring at such a high rate at this point which in turn increases caspase-3 activity. The activation of A_1 adenosine receptors at 30 minutes post-reperfusion could be too late in order to fully ensure protection. When

comparing infarct size of 2'-MeCCPA administration at the onset of reperfusion, 15 minutes post reperfusion and 30 minutes post reperfusion; the infarct size is the lowest at the point where 2'-MeCCPA is administered alone at reperfusion compared to when 2'-MeCCPA is administered alone at 30 minutes post-reperfusion. Although the decrease in infarct size at 30 minutes post-reperfusion is still significant and ensures protective effects in terms of the isolated rat heart model, it is not as protective as when 2'-MeCCPA is administered at the onset of reperfusion and at 15 minutes post-reperfusion. Therefore this could support the little difference observed in apoptosis and cleaved caspase-3 levels when 2'-MeCCPA is administered at 30 minutes post-reperfusion.

Interestingly the study conducted by Lubitz et al. (2001) administered A_3AR agonist IB-MECA 20 minutes after the onset of reperfusion. In order to understand if the defined timing of administration of the A_1 adenosine receptor agonist has an impact on the extent of cardioprotection. It would be important to explore the differences between 15 minutes, 20 minutes and 30 minutes post-reperfusion activation of A_1 adenosine receptors.

Our findings have been able to imply that the A_1 adenosine receptor, when activated at the onset of reperfusion and at 15 minutes post-reperfusion, was able to successfully confer protection; however at 30 minutes protection was limited. This protection was reversed in the presence of the A_1AR antagonist DPCPX which showed that the A_1 adenosine receptor definitely played an important role when cardioprotection was observed to the isolate rat heart model as well as the isolated rat cardiomyocytes. It was therefore useful to assess if certain pro-survival signalling pathways played a role within this protective effect that was ensured. The western blot analysis carried out within this chapter was able to successfully determine if the PI3K-AKT and MEK1/2-ERK1/2 pro-survival signalling pathways were linked with the A_1 adenosine receptor and if they played a role in the observed cardioprotection. From our findings, it can be implied that when 2'-MeCCPA (10nM) was administered at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion, there was a significant increase in p-AKT$_{(Ser473)}$ phosphorylation and this increase significantly decreased in the presence of DPCPX (200nM) at all three time points. This suggests that the protection observed throughout this chapter upon infarct size, cell death and cleaved caspase-3 activity occurred in a PI3K-AKT manner. This can be supported by a study conducted by Germack and Dickenson (2000) where the study of A_1 adenosine receptor stimulation was carried out

where A_1 adenosine agonist (1µM) increased PKB (AKT) phosphorylation and the administration of DPCPX antagonised the PKB (AKT) phosphorylation. This study was conducted upon DDT (1) MF-2 cells and although this is different to rat myocardium tissue, the trends in data still appear to be similar.

Further study by Bibli and colleagues (2014) were also able to identify that AKT was significantly upregulated when A_1AR agonist CCPA was administered to male rabbit hearts through a post-conditioning phenomenon. This shows that the RISK pathway was involved with A_1AR activation specifically which can also support this current study that has shown that the RISK pathway does play a part in A_1AR activation.

With extensive investigation put into the effects of a selective A_1 adenosine antagonist DPCPX being administered alongside an A_1 adenosine agonist 2'-MeCCPA, a non-selective adenosine antagonist, 8-SPT was also studied when administered alongside selective A_1 adenosine antagonist 2'-MeCCPA.

4.5 Summary of Findings

Within this chapter further evidence has implied that the A_1 adenosine receptor plays a vital role in cardioprotection within the isolated rat heart model which was further supported by isolated rat cardiomyocyte model. The role of the A_1 adenosine receptor was also explored when antagonised by selective A_1 adenosine antagonist DPCPX as well as unselective adenosine antagonist 8-SPT. These effects were all explored at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion. Our results showed that:

- The administration of the A_1AR agonist 2'-MeCCPA (10nM) implies that cardioprotection occurred. This was observed though the limited infarct effect, a decrease in cell death and a decrease in cleaved caspase-3 activity. These effects were observed when 2'-MeCCPA was administered at the onset of reperfusion/reoxygenation, 15 minutes and 30 minutes post-reperfusion/reoxygenation.
- The protective effects observed were significantly antagonised with the administration of selective A_1 adenosine antagonist, DPCPX. When 2'-MeCCPA was

administered alongside 2'-MeCCPA at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion; infarct limiting effects were antagonised and cell death increased. Showing the importance of the activation of the A_1 adenosine receptor at the onset of reperfusion and 15 minutes into the onset of reperfusion. When 2'-MeCCPA and DPCPX was co-administered at 30 minutes post-reperfusion, there was no effect upon caspase-3 activity. Summarising that delayed activation of A_1 adenosine receptors at 30 minutes post-reperfusion may be too late for activation.

- Western blot analysis highlighted an increase of p-AKT $_{(Ser473)}$ when 2'-MeCCPA was administered at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion however the administration of DPCPX at these different time-points alongside 2'-MeCCPA antagonised the phosphorylation of these proteins. This highlighted the importance of activation and delayed activation of A_1 adenosine receptors and the recruitment of RISK pro-survival signalling pathway which includes the PI3K-AKT and MEK1/2-ERK1/2 cell signalling pathways.
- Selective A_1 adenosine agonist 2'-MeCCPA was administered alongside unselective adenosine antagonist 8-SPT and it was found that when both agonist and antagonist were administered together, there was an increase in infarct effect at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion.
- An increase in cell death was observed when 2'-MeCCPA and 8-SPT were administered together in comparison to when 2'-MeCCPA was administered alone showing that 8-SPT blocked 2'-MeCCPA mediated cardioprotection. No difference in cell death was detected at 15 minutes and 30 minutes post-reperfusion.
- An increase in cleaved caspase-3 activity was also observed when 2'-MeCCPA and 8-SPT was administered together in comparison to when 2'-MeCCPA was administered alone at the onset of reperfusion and at 15 minutes post-reperfusion. No difference was detected when both agonist and antagonist was administered together at 30 minutes post-reperfusion.

Chapter 5: Administration of A_1 adenosine receptor agonist, 2'-MeCCPA (10nM) at the onset of reperfusion compared to 15 minutes and 30 minutes post-reperfusion to protect the myocardium from ischaemia-reperfusion injury via the recruitment of the PI3K-AKT cell signalling pathway

5.1 Introduction

PI3K is a serine/threonine kinase and this plays a major role in regulating cell growth, differentiation and survival (Ban et al. 2008). PI3K is directly linked to the reperfusion injury salvage kinase (RISK) pathway and has been shown to be important in the intracellular signalling pathway required for cardioprotection. Studies conducted by Hausenloy et al. (2007) have undertaken detailed research into how activation of the PI3K-AKT cell signalling pathway can enhance cardioprotection; but until now there is limited research on the role of PI3K in the post-reperfusion A_1 adenosine receptor mediated cardioprotection.

The phosphorylation of PI3K can subsequently lead to phosphorylation of AKT (also known as protein kinase B). This cascade can therefore further lead to cardioprotective effects within a myocardial ischaemia-reperfusion setting (Jonassen et al. 2004; Mangi et al. 2003; Effendi et al. 2020). AKT, which is made up of serine and threonine b kinases, was discovered around 25 years ago and has also been an important focus of many studies within the fields of biology and medicine since (Manning and Toker 2017). Studies conducted on mouse and human genetics have shown the physiological roles of the AKT network.

AKT has been shown to play a key role when it comes to the induction of cardioprotection through ischaemic preconditioning (Lai et al. 2015; Singh et al. 2018). Links have been proposed to suggest that adenosine preconditioning also promotes AKT phosphorylation in order to trigger cardioprotection. A recent study conducted by Shao et al. (2017) reported that pre-treatment with A_1 adenosine receptor agonist N6-cyclohexyladenosine had the ability to trigger cardioprotection against ischaemia-reperfusion injury via the increase in phosphorylation of AKT (Shao et al. 2017). The A_1 and the A_3 receptors play a major role in intracellular signalling in order to trigger cardioprotection (Cohen and Downey 2007).

The phenomenon of reperfusion is a prerequisite in order to salvage the viable myocardium following an acute myocardium infarction; however it is not without risk that the act of reperfusion can paradoxically result in myocyte death alongside (Hausenloy and Yellon 2004). This is termed lethal reperfusion-induced injury. Therapeutic strategies can be adhered to in order to target and attenuate reperfusion-induced cell death by providing pharmacological agents to limit myocardial infarction (Hausenloy and Yellon 2004). Recent evidence has implied that apoptotic cell death can be implicated during the phase of reperfusion to contribute to lethal reperfusion-induced injury and targeting the cellular anti-apoptotic mechanisms at the time of reperfusion is therefore a potential approach in being able to attenuate reperfusion-induced cell death (Hausenloy and Yellon 2004). It has been shown that the activation of anti-apoptotic cell survival kinase signalling cascades such as the PI3K-AKT pathway has been implicated in conferring cardioprotection at the time of reperfusion and has limited infarct size (Hausenloy and Yellon 2004).

With the pro-survival PI3K-AKT kinase cascade mediating cardioprotection at the time of reperfusion through the association of ischaemic preconditioning, evidence suggests the recruitment of this pro-survival pathway can occur at the time of reperfusion itself and cause cardioprotective qualities (Hausenloy and Yellon 2004).

5.1.1 Aims and Objectives

1. To determine the cardioprotective effects of the A_1 adenosine receptor agonist, 2'-MeCCPA (10nM) in association with the recruitment of the pro-survival PI3K-AKT cell signalling pathway. A_1AR agonist 2'-MeCCPA (10nM) was administered at the onset of reperfusion in the presence and absence of PI3K inhibitor Wortmannin in order to assess infarct size (%), cell death (apoptosis and necrosis), p-AKT phosphorylation in Western blot and caspase-3 activity.
2. To determine the cardioprotective effects of the A_1 adenosine receptor agonist, 2'-MeCCPA (10nM) in association with the post-reperfusion/reoxygenation recruitment of the pro-survival PI3K-AKT cell signalling pathway. A_1AR agonist 2'-MeCCPA (10nM) was administered at 15 minutes post-reperfusion/reoxygenation in the presence and

absence of PI3K inhibitor Wortmannin in order to assess infarct size (%), cell death (apoptosis and necrosis), p-AKT phosphorylation and caspase-3 activity.

3. To determine the effects of A_1AR agonist 2'-MeCCPA (10nM) in the association of with the post-reperfusion/reoxygenation recruitment of the pro-survival PI3K-AKT cell signalling pathway. 2'-MeCCPA (10nM) was administered at 30 minutes post-reperfusion/reoxygenation in the presence and absence of PI3K inhibitor Wortmannin in order to assess infarct size (%), cell death (apoptosis and necrosis), p-ERK phosphorylation and caspase-3 activity.

5.2 Methods

5.2.1 Chemicals

2'-MeCCPA and Wortmannin were both supplied from Tocris Cookson (Bristol) and prepared in the same manner as described in Chapter 2, Section 2.2.

5.2.2 Animals

Adult male Sprague-Dawley rats (350 ± 50g) were supplied from Charles River (UK). Animals all received human care and assistance and were sacrificed by cervical dislocation as outlined in the Schedule 1 Home Office Procedure in accordance with the Scientific Procedure Act 1986. This process is described in Chapter 2, Section 2.1.

5.2.3 Langendorff protocol – Isolated perfused rat heart preparation

Briefly, experiments using the Langendorff technique were carried out for 175 minutes in total (full details in Chapter 2, Section 2.3.5). Hearts were allowed a 20 minutes stabilisation period where hearts were perfused with KH buffer, followed by 35 minutes of simulated regional ischaemia followed by 120 minutes of reperfusion. Hearts were randomly allocated to the following control and treatment groups:

a) Normoxic control – Rat hearts perfused with KH buffer for 175 minutes (no simulated ischaemia induced).

b) Ischaemia-reperfusion (IR) control – Rats hearts were perfused with KH buffer for 20 minutes followed by 35 minutes of simulated regional ischaemia and 120 minutes of reperfusion.
c) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of PI3K inhibitor Wortmannin (100nM) at the onset of reperfusion – Rats were perfused with KH buffer for 20 minutes, 35 minutes of simulated regional ischaemia followed by 120 minutes of reperfusion where drug administration occurred at the onset of reperfusion.
d) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of PI3K inhibitor Wortmannin (100nM) at 15 minutes post-reperfusion - Rats were perfused with KH buffer for 20 minutes, 35 minutes of simulated regional ischaemia followed by 120 minutes of reperfusion where drug administration occurred at 15 minutes post-reperfusion.
e) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of PI3K inhibitor Wortmannin (100nM) at 30 minutes post-reperfusion - Rats were perfused with KH buffer for 20 minutes, 35 minutes of simulated regional ischaemia followed by 120 minutes of reperfusion where drug administration occurred at 30 minutes post-reperfusion.

Throughout the course of the Langendorff experiments, the stability of the hearts were consistently monitored by the heart rate (HR), left ventricular developed pressure (LVDP) and coronary flow (CF) haemodynamic parameters (as described in Chapter 2, Section 2.3.2).

After the reperfusion period, the infarct size to risk ratio was assessed with the use of Evans blue and triphenyltetrazolium chloride (TTC) staining procedures as described in Chapter 2, Section 2.3.6.

5.2.4 Isolation of adult rat ventricular cardiomyocytes

Isolation of adult rat ventricular cardiomyocytes was previously described in Chapter 2, Section 2.4.

5.2.5 Induction of hypoxia and reoxygenation conditions in adult rat cardiomyocytes

Fully described in Chapter 2, Section 2.4.1.

5.2.6 Experimental drug treatment protocol in adult rat ventricular cardiomyocytes

The isolated rat cardiomyocytes were exposed to differing control and drug treatments. All experimental conditions are detailed as below:

a) Normoxic control – Isolated myocytes were exposed to normoxic conditions for a total of 4 hours at 37^{o}C, 5% CO_2 and 95% O_2.
b) Hypoxia-Reoxygenation control – Isolated cardiomyocytes were exposed to 1 hour of hypoxia conditions followed by the onset of reoxygenation for 3 hours.
c) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of PI3K inhibitor Wortmannin (100nM) at the onset of reoxygenation – Isolated cardiomyocytes were exposed to 1 hour hypoxia conditions followed by drug treatment at the onset of reoxygenation for 3 hours at 37^{o}C, 5% CO_2 and 95% O_2.
d) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of PI3K inhibitor Wortmannin (100nM) at 15 minutes post-reoxygenation – Isolated cardiomyocytes were exposed to 1 hour hypoxia conditions followed by drug treatment at 15 minutes post-reoxygenation for 3 hours at 37^{o}C, 5% CO_2 and 95% O_2.
e) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of PI3K inhibitor Wortmannin (100nM) at 30 minutes post-reoxygenation – Isolated cardiomyocytes were exposed to 1 hour hypoxia conditions followed by drug treatment at 30 minutes post-reoxygenation for 3 hours at 37^{o}C, 5% CO_2 and 95% O_2.

5.2.7 Quantitative Analysis of Cell Death using Dead Cell Apoptosis Kit with Annexin V FITC with Propidium Iodide (PI) in Adult Rat Cardiomyocytes

Followed by the drug treatments described above, isolated rat ventricular cardiomyocytes were assess for their levels of apoptosis and necrosis using the Dead Cell Apoptosis Kit with Annexin V FITC and PI was purchased from ThermoFisher (UK) (previously detailed in Chapter 2, Section 2.5.2). Data was normalised against the cell only control and the values obtained were calculated as a relative change in apoptosis and necrosis activity of the mean absorbance of the control group.

5.2.8 Analysis of Cleaved Caspase-3 Activity in Adult Rat Cardiomyocytes

Following the drug treatment protocol as described above, the rat cardiomyocytes were probed with cleaved caspase-3 antibody as described in Chapter 2, Section 2.5.1. Data obtained was normalised to the cell only control by subtracting the mean fluorescence background recorded in untreated samples. Data was presented as a relative change in fluorescence activity.

5.2.9 Western blot analysis

Western blot analysis was performed as described in Chapter 2, Section 2.6. Following the stages of separation and protein transfer procedure; membrane blots were probed for phosphorylated and total form of the monoclonal rabbit AKT $_{(Ser473)}$ protein (Cell Signalling, UK) as stated in Section 2.6.7. Protein detection was quantified with the use of Super Signal West Femto Maximum Substrate Solution (ThermoFisher Scientific, UK) to perform an enhanced chemi-luminescence assay (as detailed in Chapter 2, Section 2.6.8) and proteins bands were visualised with the use of Bio-Rad Quantity One programme. The relative variations of the levels of phosphorylated monoclonal rabbit AKT $_{(Ser473)}$ protein were normalised to the total form of the monoclonal rabbit AKT protein. GAPDH was also used as an internal loading control within all experiments as explained in Chapter 2, Section 2.6.7.

5.2.10 Data Analysis

All data that was presented in this project is expressed at the mean ± standard error of the mean (SEM). IBM Statistical Package for Social Sciences (SPSS®) software was used to statistically analyse the data. The statistical tests currently used to analyse infarct sizes, band densities and cell population data was by one-way ANOVA accompanied by Fishers Protected Least Significant Difference (LSD) test for multiple comparisons. To assess the difference in the data sets, a p-value of $p<0.05$ was used to consider statistical significance.

Microsoft Excel was also used to present all data graphically.

5.3 Results

5.3.1 Profiling the effects of the administration of 2'-MeCCPA (10nM) at the onset of reperfusion/reoxygenation in the presence and absence of PI3K-AKT inhibitor, Wortmannin (100nM) and its affects upon isolated rat myocardium model and isolated rat cardiomyocytes.

5.3.1.1 Profiling the effects of 2'-MeCCPA in the presence and absence of PI3K-AKT inhibitor, Wortmannin (100nM) on haemodynamic parameters (left ventricular developed pressure, heart rate and coronary flow) at the onset of reperfusion.

All hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and a further 120 minutes of reperfusion where 2'-MeCCPA (10nM) was administered in the presence and absence of Wortmannin (100nM) (PI3K-AKT inhibitor). Wortmannin (100nM) was also administered alone also. All treatments were administered at the onset of reperfusion to profile the effects of this inhibitor upon the A_1 adenosine receptor agonist, 2'-MeCCPA (10nM).

No significant difference between the groups at any of the time-points within the reperfusion period was observed ($p>0.05$) (Figure 5.1). Throughout the ischaemic period, all treatment groups had a significantly decreased LVDP in comparison to the normoxic control ($p<0.05$) (Figure 5.1).

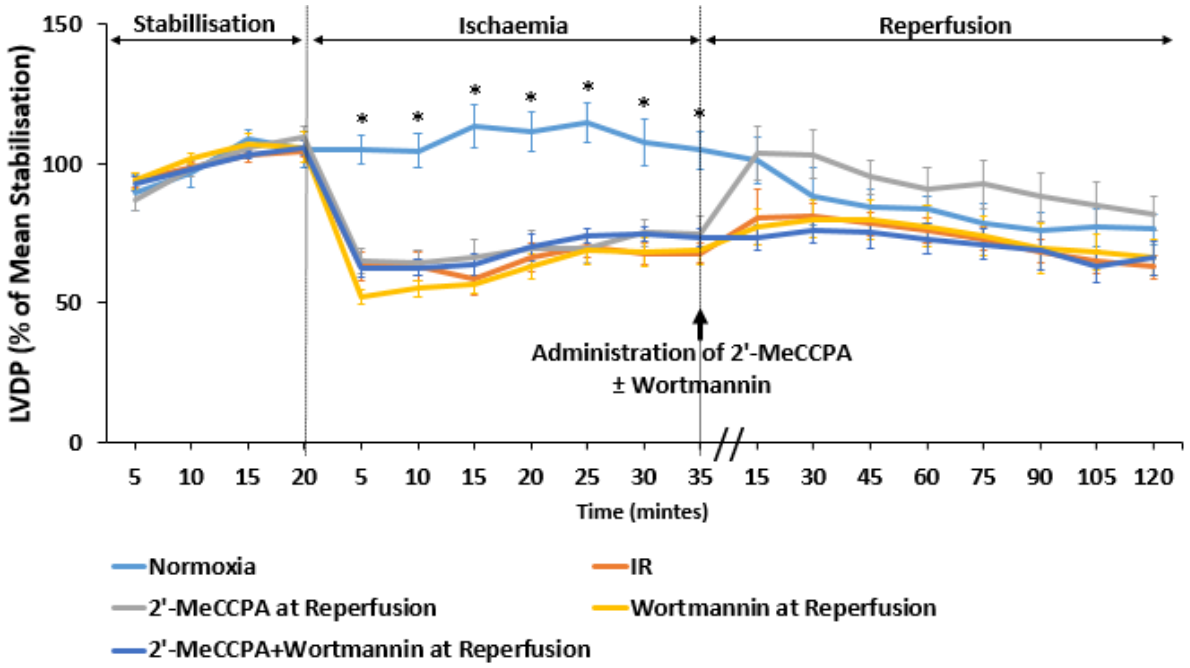


Figure 5. 1 Effects of 2'-MeCCPA (10nM) in the presence and absence of Wortmannin (100nM) on the left ventricular developed pressure (LVDP) within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at the onset of reperfusion in the presence and absence of Wortmannin (100nM). Data was presented as Mean±SEM, n=6-8. * $p<0.05$ vs. Normoxia.

There was no significant changes detected in the heart rate between all time-matched treatment groups ($p>0.05$) (Figure 5.2).

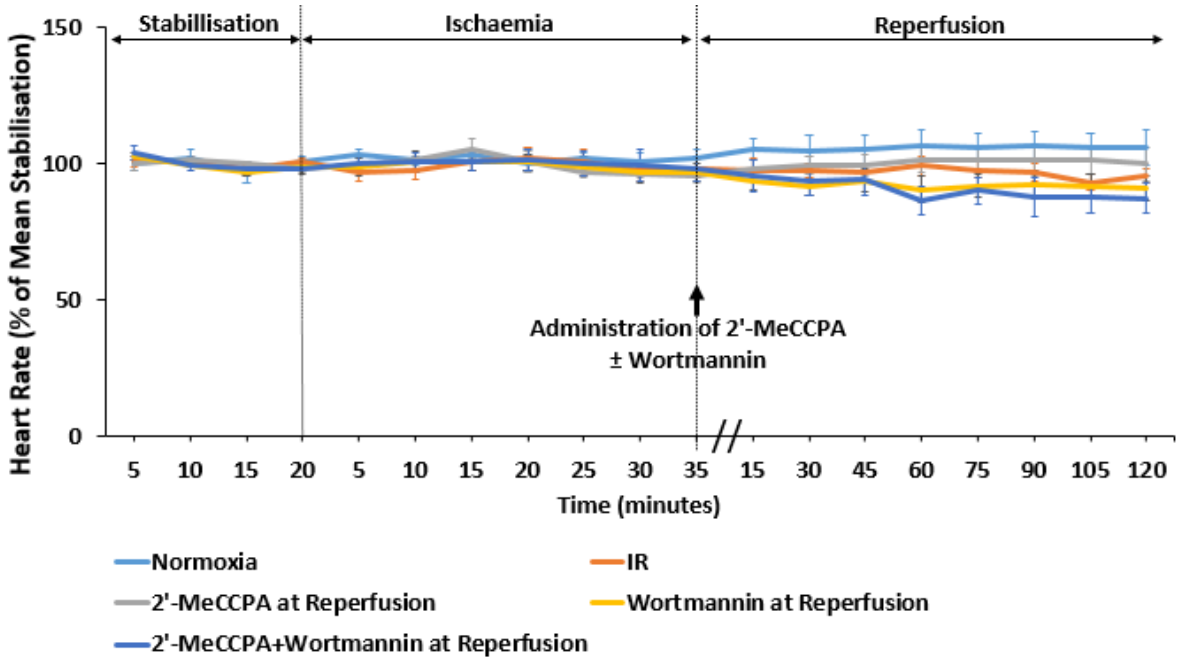


Figure 5. 2 Effects of 2'-MeCCPA (10nM) in the presence and absence of Wortmannin (100nM) on the heart rate within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at the onset of reperfusion in the presence and absence of Wortmannin (100nM). Data was presented as Mean±SEM, n=6-8.

No significant changes were observed between treatment groups at each time-matched points within the reperfusion period ($p>0.05$) (Figure 5.3). Although within the ischaemic period, all time-matched treatment groups had a significantly lower coronary flow in comparison to the normoxic control ($p<0.05$) (Figure 5.3).

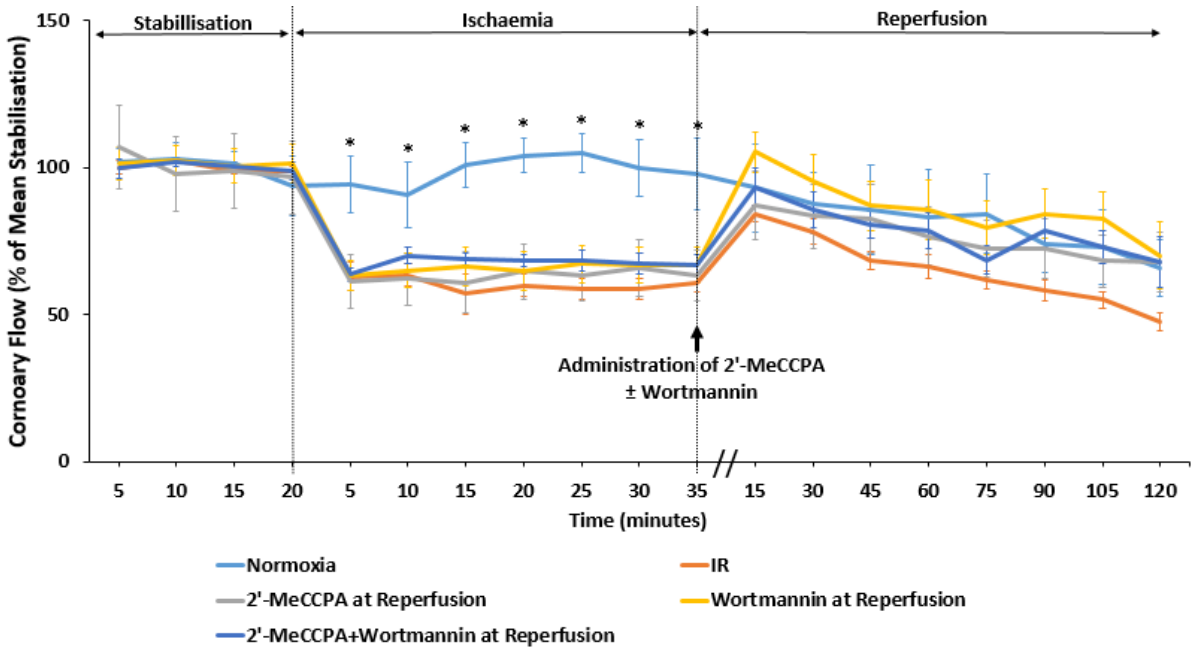


Figure 5. 3 Effects of 2'-MeCCPA (10nM) in the presence and absence of Wortmannin (100nM) on the coronary flow within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at the onset of reperfusion in the presence and absence of Wortmannin (100nM). Data was presented as Mean±SEM, n=6-8. * $p<0.05$ vs. Normoxia.

5.3.1.2 Profiling the effects of 2'-MeCCPA (10nM) in the presence and absence of PI3K-AKT signalling pathway inhibitor, Wortmannin (100nM) on infarct size and risk ratio (%) in isolated hearts subjected to ischaemia reperfusion injury at the onset of reperfusion

All hearts were subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. A_1 adenosine receptor agonist, 2'-MeCCPA (10nM), was firstly administered alone or in the presence of PI3K signalling pathway inhibitor, Wortmannin (100nM). All treatments were administered at the onset of reperfusion to profile their effects on infarct size to risk ratio (%).

When 2'-MeCCPA (10nM) was administered alone, a significant decrease in infarct size to risk ratio (%) was observed compared to the IR control (28 ± 4% vs. 55 ± 6%, $p<0.001$) (Figure 5.4). A significant increase in infarct size to risk ratio was detected (%) when Wortmannin (100nM) + 2'-MeCCPA (10nM) was administered in conjunction compared with when 2'-MeCCPA

(10nM) was administered alone at the onset of reperfusion (65 ± 7% vs. 28 ± 4%, p<0.001) (Figure 5.4). No significant effect was observed when Wortmannin (100nM) was administered alone (p>0.05) (Figure 5.4).

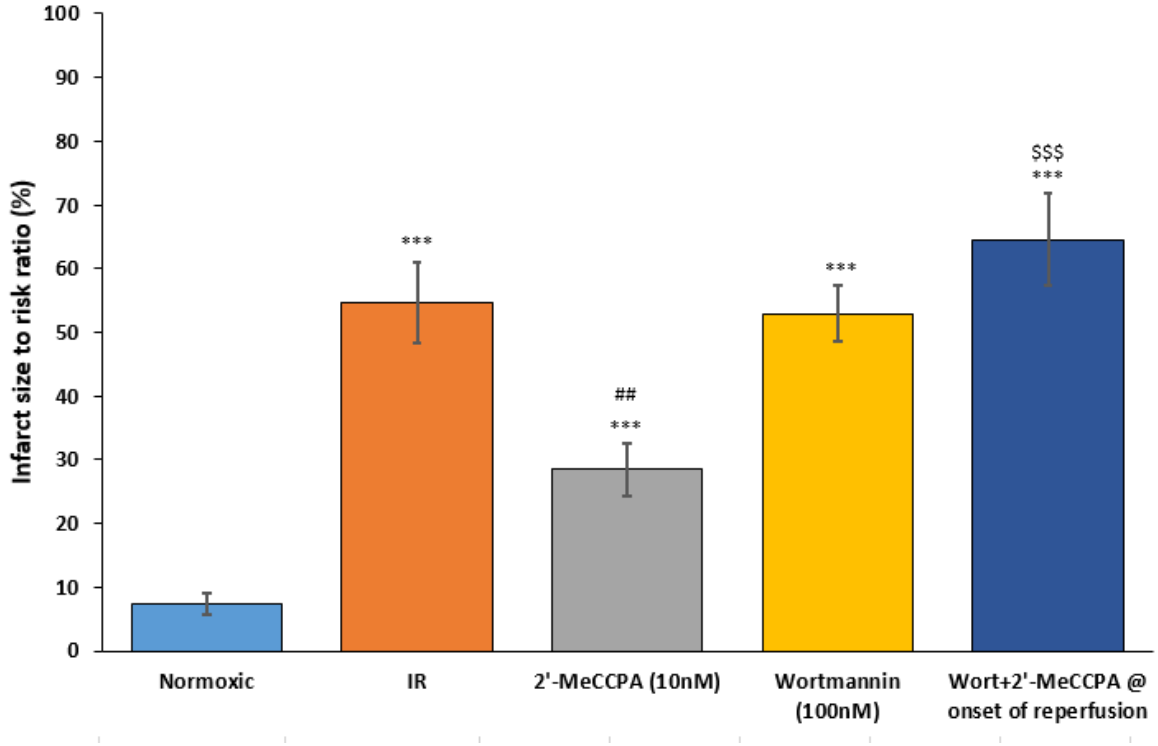


Figure 5. 4 Infarct size to risk ratio (%) within isolated perfused hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion subjected to 2'-MeCCPA (10nM) administered alone as well as Wortmannin (100nM) (PI3K-AKT signalling pathway inhibitor) administered alone and 2'-MeCCPA + Wortmannin co-administered together all at the onset of reperfusion. Data presented as Mean±SEM. n=6-8. *** p<0.001 vs. normoxic, ## p<0.01 vs. IR, $$$ p<0.001 vs. 2'-MeCCPA.

5.3.1.3 The role of PI3K-AKT cell signalling pathway in 2'-MeCCPA mediated cardioprotection when administered at the onset of reperfusion to isolated rat cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation and its effect upon on apoptosis and necrosis.

It has previously been elucidated within this chapter that when 2'-MeCCPA (10nM) is administered at the onset of reoxygenation there is significant protection of the rat cardiac myocytes from hypoxia-reoxygenation injury via anti-apoptotic and anti-necrotic manners.

In order to identify the anti-apoptotic as well as the anti-necrotic mechanisms within this section, the role of the PI3K-AKT cell signalling pathway was explore using Wortmannin

(100nM) alongside 2'-MeCCPA (10nM). Isolated myocytes were subjected to 1 hour of hypoxia and 3 hours of reoxygenation where A_1 adenosine receptor agonist, 2'-MeCCPA (10nM) was administered at the onset of reoxygenation in the presence and absence of PI3K-AKT inhibitor Wortmannin (100nM).

The administration of A_1 agonist 2'-MeCCPA (10nM) in the presence of PI3K-AKT inhibitor Wortmannin (100nM) significantly abolished the anti-apoptotic characteristics detected when 2'-MeCCPA (10nM) when administered alone throughout reoxygenation (29±4% 2'-MeCCPA + Wortmannin vs. 17±4% 2'-MeCCPA, $p<0.001$) (Figure 5.5).

Administration of 2'-MeCCPA (10nM) in the presence of PI3K-AKT inhibitor Wortmannin also significantly abolished the anti-necrotic effects of when 2'-MeCCPA (10nM) was administered alone throughout reoxygenation (25±3% 2'-MeCCPA + Wortmannin vs. 13±2% 2'-MeCCPA, $p<0.001$).

No significant effect was observed when Wortmannin (100nM) was administered alone throughout reoxygenation both on cellular apoptosis and necrosis ($p>0.05$) (Figure 5.5).

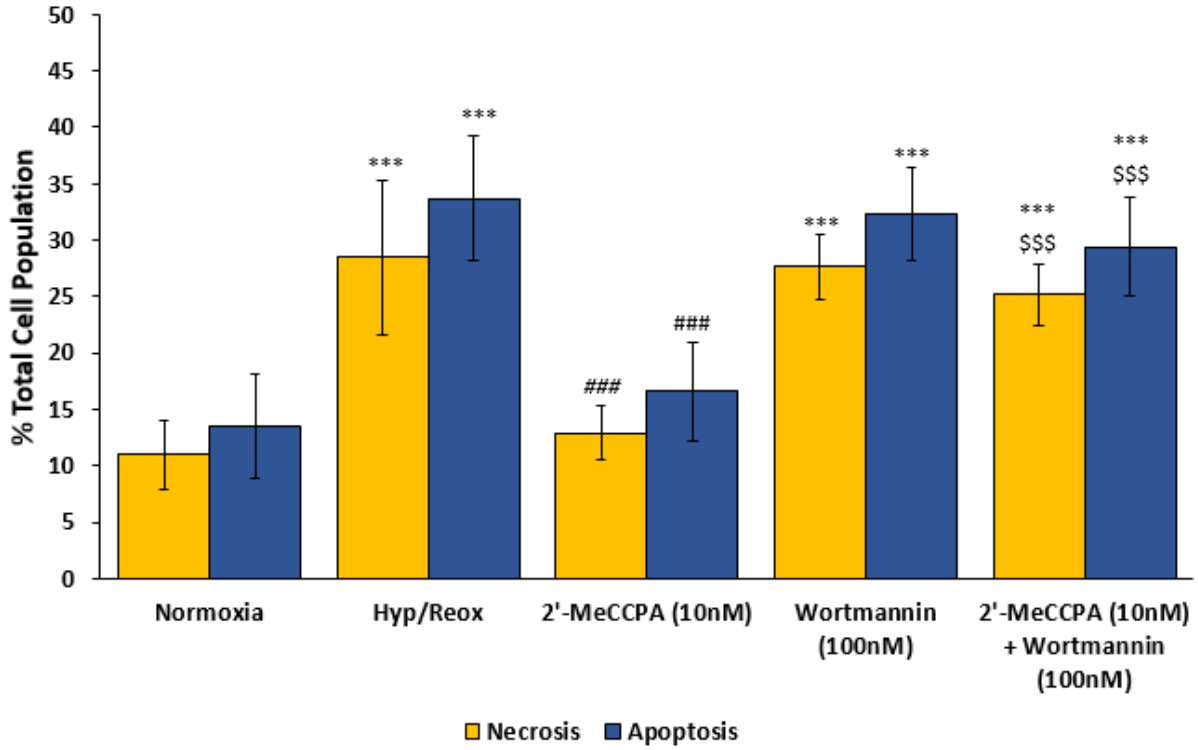


Figure 5. 5 Assessment of apoptosis and necrosis within isolated rat cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation. 2'-MeCCPA (10nM) was administered at the onset of reoxygenation in the presence and absence of PI3K-AKT inhibitor, Wortmannin (100nM). Results were shown as Mean±SEM and were expressed as a percentage of 10 000 cells counted from 6 individual experiments. ***p<0.001 vs. Normoxia. ### p<0.001 vs. Hyp/Reox. $$$ p<0.001 vs. 2'-MeCCPA.

5.3.1.4 Profiling effects of 2'-MeCCPA (10nM) when administered at reperfusion on AKT phosphorylation at the onset of reperfusion

It was shown that 2'-MeCCPA (10nM) was able to protect the myocardium from ischaemia/reperfusion injury within isolated perfused rat hearts where this same protection was abolished in the presence of PI3K inhibitor Wortmannin (100nM) (Figure 5.6). In order to understand the signalling pathways involved when 2'-MeCCPA (10nM) mediated cardioprotection when administered at the onset of reperfusion meant it was necessary to investigate the phosphorylation activity of the protein $AKT_{(ser473)}$ at the onset of reperfusion in the presence and absence of Wortmannin (100nM).

It was found that phosphorylation of $AKT_{(ser473)}$ was observed within the non-treated control as well as within 2'-MeCCPA (10nM) treated hearts. At the onset of reperfusion, 2'-MeCCPA (10nM) was administered and continued to be administered throughout the 10 minutes of reperfusion of this protocol (as mentioned in the methodology section) in the presence of A_1 adenosine agonist 2'-MeCCPA (10 nM), there was a significant increase in phosphorylation of $AKT_{(ser473)}$ ($p<0.01$) compared to the time matched control hearts (Figure 5.6).

The upregulation of $AKT_{(ser473)}$ phosphorylation observed by 2'-MeCCPA (10nM) after 10 minutes of reperfusion was significantly reduced in the presence of the PI3K inhibitor, Wortmannin (100nM) ($p<0.001$) (Figure 3.37). When Wortmannin (100nM) was administered alone at the onset of reperfusion, there was no significant effect upon $AKT_{(ser473)}$ phosphorylation to the time matched non-treated control hearts ($p>0.05$) (Figure 5.6).

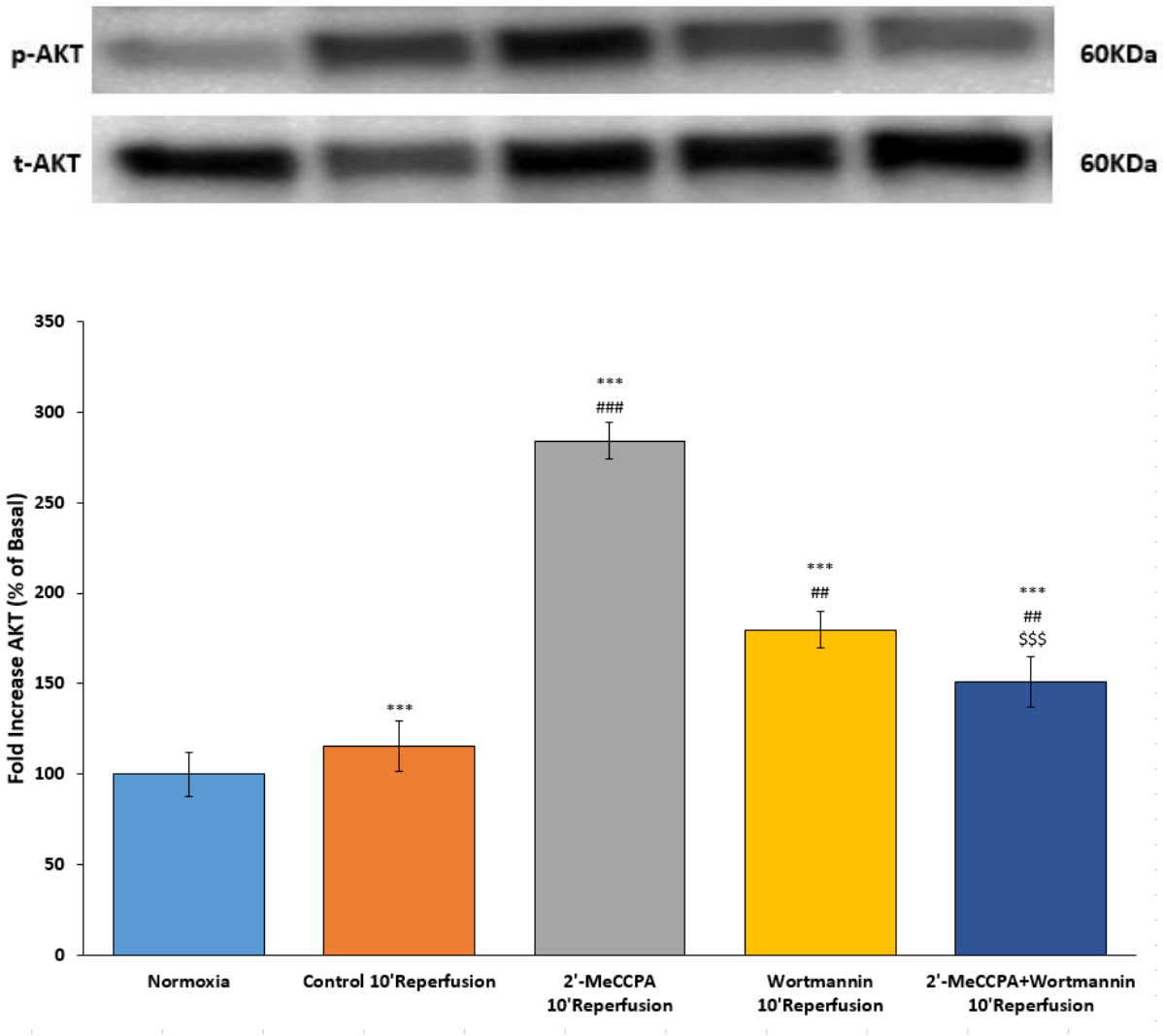


Figure 5. 6 The assessment of $AKT_{(ser473)}$ phosphorylation within isolated hearts subjected to 65 minutes perfusion (Normoxia) or 20 minutes stabilisation, 35 minutes ischaemia followed by 10 minutes of reperfusion for non-treated control (Control 10'Reperfusion). The A_1 adenosine agonist 2'-MeCCPA (10nM) was administered at the onset of reperfusion in the presence and absence of PI3K inhibitor Wortmannin (100nM) for the duration of 10 minutes of reperfusion. Results are shown as Mean±SEM of four individual experiments. *** p<0.001 vs. Normoxia. ### p<0.001 vs. Control 10'Reperfusion. ## p<0.01 vs. Control 10'Reperfusion. $$$ p<0.001 vs. 2'-MeCCPA 10'Reperfusion.

5.3.1.5 Profiling the role of PI3K-AKT cell signalling pathway on cleaved-caspase 3 activity upon the administration of 2'-MeCCPA (10nM) at the onset of reoxygenation

The role of the PI3K-AKT cell signalling pathway on cleaved-caspase 3 was determined in terms of 2'-MeCCPA (10nM) mediated cardioprotection. Isolated adult rat myocytes underwent 1 hour of hypoxia and 3 hours of reoxygenation where the A_1 adenosine receptor agonist 2'-MeCCPA (10nM) was administered in the presence and absence of PI3K-AKT inhibitor, Wortmannin (100nM) throughout the reoxygenation period.

It was found that the administration of 2'-MeCCPA (10nM) throughout the reoxygenation period significantly decreased cleaved-caspase 3 activity; and in the presence of PI3K inhibitor Wortmannin (100nM), this decrease in cleaved-caspase 3 was abolished (181±35% 2'-MeCCPA at onset of Reox vs. 258±18% 2'-MeCCPA + Wortmannin at onset of reoxygenation, $p<0.01$) (Figure 5.7). Administration of Wortmannin (100nM) alone throughout the duration of reoxygenation had no significant effect upon cleaved-caspase 3 activity compared with the Hyp/Reox control group (282±47% Wortmannin at onset of reoxygenation vs. 313±35% Hyp/Reox, $p>0.05$) (Figure 5.7).

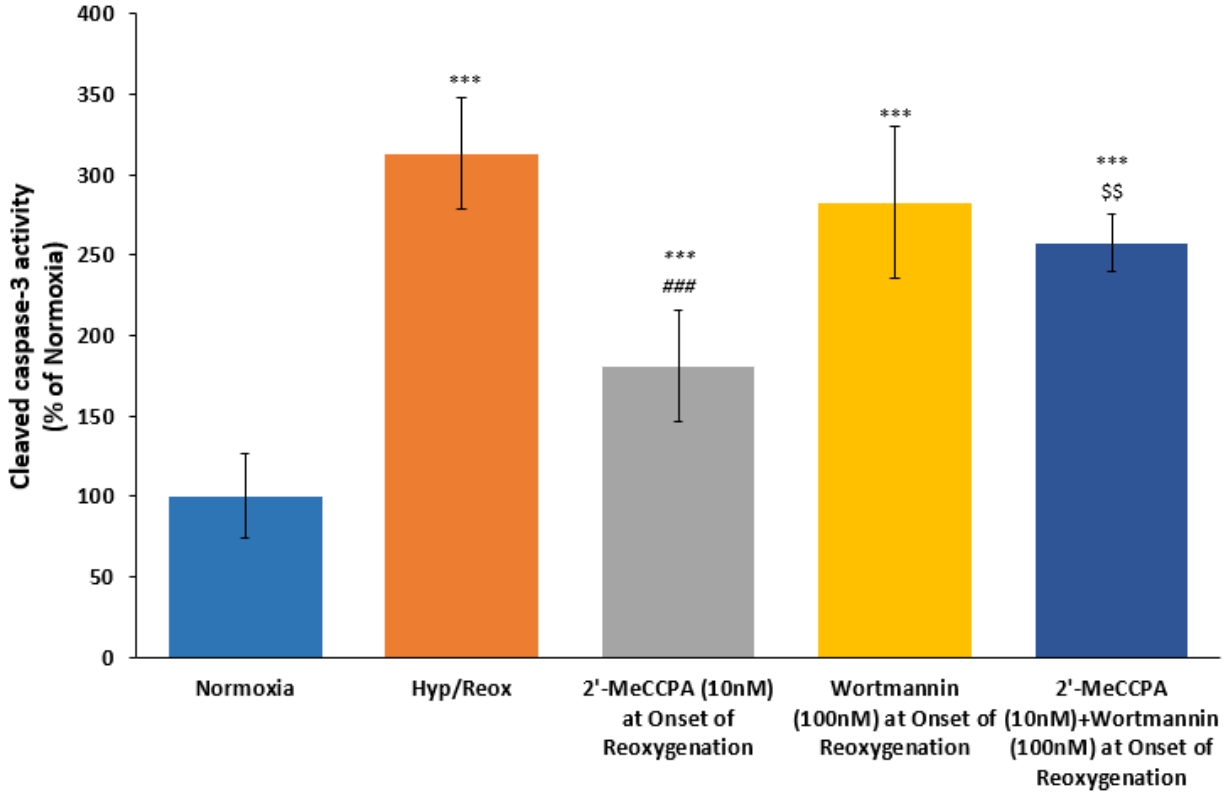


Figure 5. 7 Cleaved-caspase 3 activity within isolated rat cardiomyocytes subjected to 4 hours of reoxygenation (Normoxia) or 1 hour of hypoxia followed by 3 hours of reoxygenation (Hyp/Reox). 2'-MeCCPA (10nM) was administered at the onset of reoxygenation in the presence and absence of PI3K-AKT cell signalling inhibitor Wortmannin (100nM). Mean±SEM on 6 individual experiments. *** $p<0.001$ vs. Normoxia. ### $p<0.001$ vs. Hyp/Re. $$ $p<0.01$ 2'-MeCCPA @onset of Reox.

5.3.2 Profiling the effects of the administration of 2'-MeCCPA (10nM) at 15 minutes or 30 minutes post-reperfusion/reoxygenation in the presence and absence of PI3K-AKT inhibitor, Wortmannin (100nM) and its affects upon isolated rat myocardium model and isolated rat cardiomyocytes.

5.3.2.1a Effects of postponing the administration of 2'-MeCCPA (10nM) to 15 minutes post-reperfusion in the presence and absence of PI3K-AKT inhibitor, Wortmannin (100nM) on haemodynamic parameters (left ventricular developed pressure (LVDP), heart rate and coronary flow).

Throughout the Langendorff studies, the left ventricular developed pressure, heart rate and coronary flow was consistently monitored. Hearts were subjected to 20 minutes stabilisation, 35 minutes of ischaemia followed by 120 minutes of reperfusion with the administration of 2'-MeCCPA (10nM) in the presence and absence of Wortmannin (100nM) at 15 minutes post reperfusion.

It was observed that throughout ischaemia, there was a significant decrease in LVDP of all treatment groups at time matched points in comparison to the normoxia control group ($p<0.05$) (Figure 5.8). Throughout the period of reperfusion, there was a general decline in LVDP after 15 minutes of reperfusion in all treatment groups, these changes were not significant ($p>0.05$) (Figure 5.8).

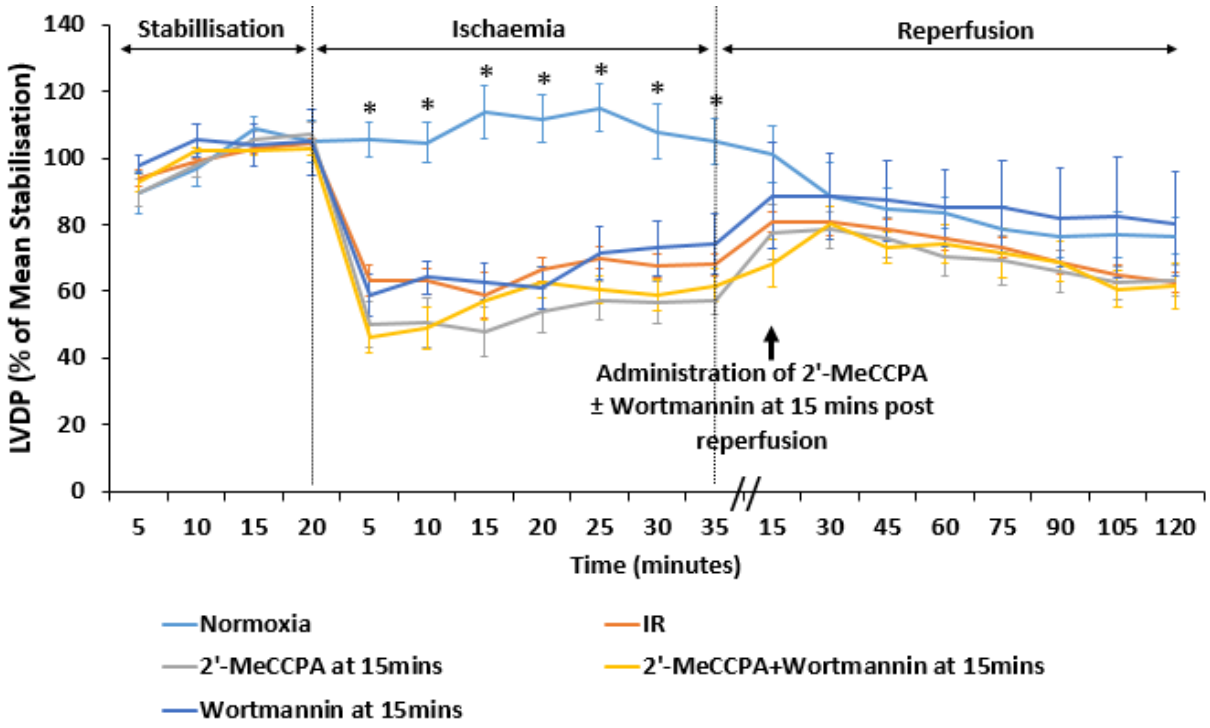


Figure 5. 8 Assessing the effects of 2'-MeCCPA (10nM) in the presence and absence of Wortmannin (100nM) when administered at 15 minutes post reperfusion on left ventricular developed pressure (LVDP) within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Data was presented as Mean±SEM, n=6-8. * $p<0.05$ All groups vs. Normoxia (Ischaemia).

There was no significant effects detected on heart rate between all time-matched treatment groups ($p>0.05$) (Figure 5.9).

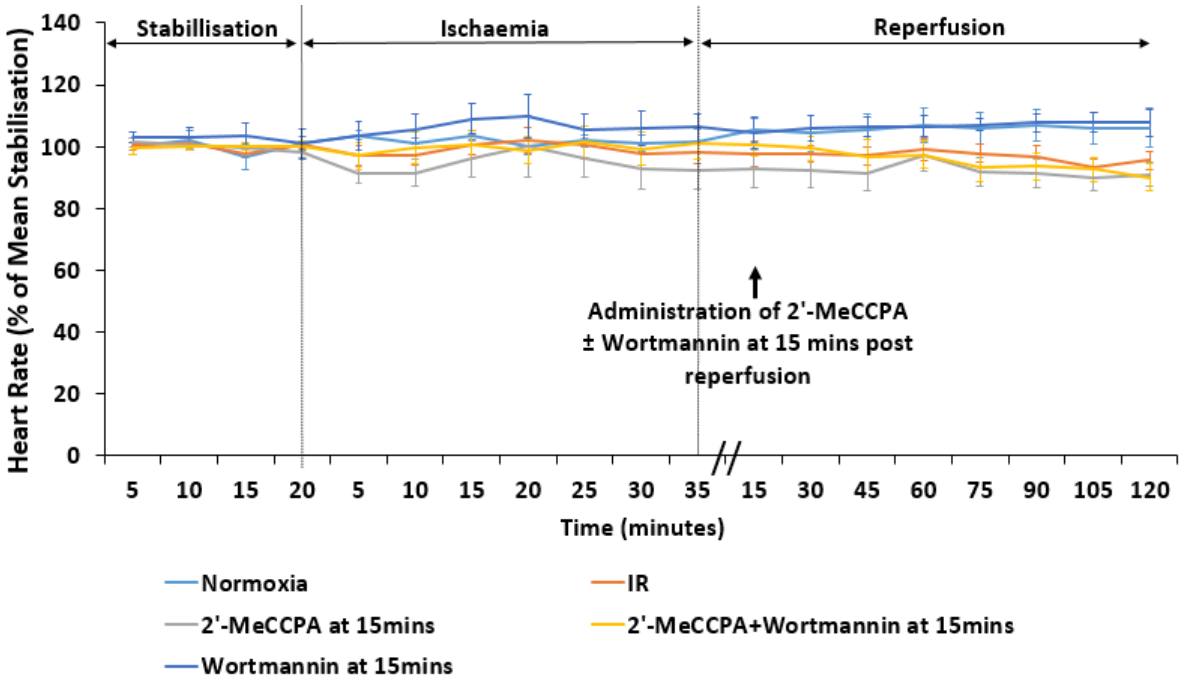


Figure 5. 9 The effects of 2'-MeCCPA (10nM) in the presence and absence of Wortmannin (100nM) when administered at 15 minutes post reperfusion on heart rate within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Data was presented as Mean±SEM, n=6-8.

Within the ischaemic period of the Langendorff protocol, it was observed that the coronary flow for all treatment groups at all time-matched points significantly decreased in comparison to the normoxic control group ($p<0.05$) (Figure 5.3). No significant changes were observed between the treatment groups at each time matched points within the reperfusion period ($p>0.05$) (Figure 5.10).

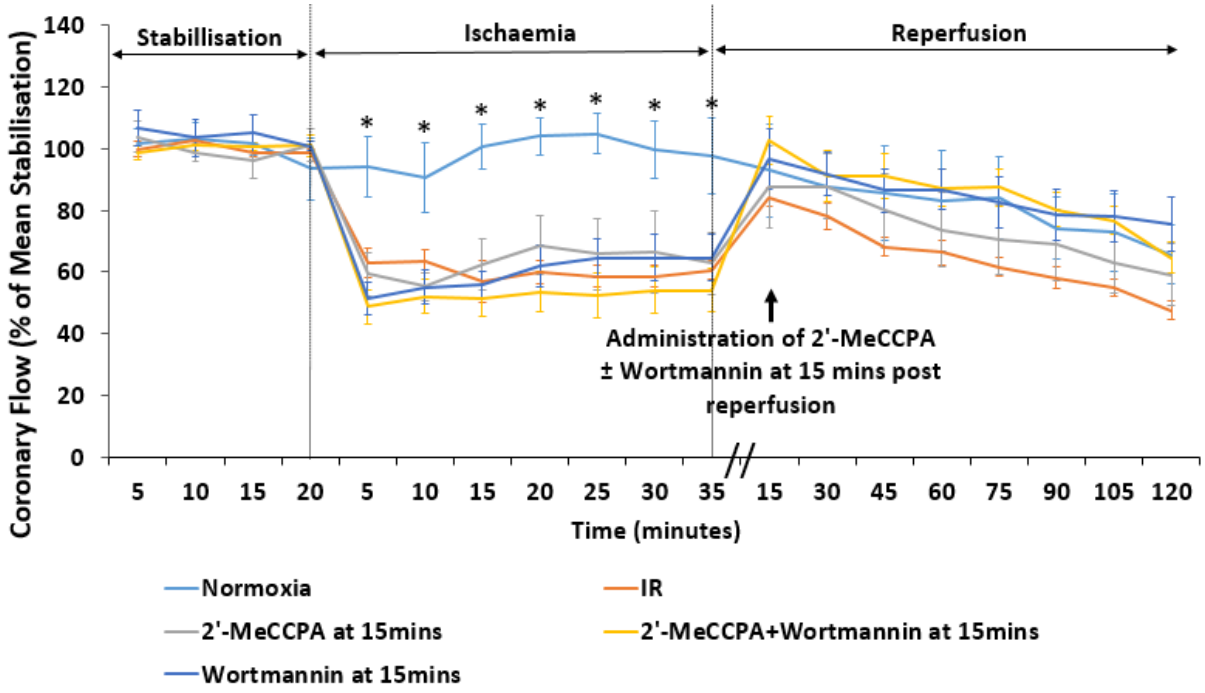


Figure 5. 10 The effects of 2'-MeCCPA (10nM) in the presence and absence of Wortmannin (100nM) when administered at 15 minutes post reperfusion on coronary flow within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Data was presented as Mean±SEM, n=6-8. * $p<0.05$ vs. All groups vs. Normoxia (in Ischaemia).

5.3.2.1b Effects of postponing the administration of 2'-MeCCPA (10nM) to 30 minutes post-reperfusion in the presence and absence of PI3K-AKT inhibitor, Wortmannin (100nM) on haemodynamic parameters (left ventricular developed pressure (LVDP), heart rate and coronary flow).

Throughout the Langendorff studies, the left ventricular developed pressure, heart rate and coronary flow was consistently monitored. Hearts were subjected to 20 minutes stabilisation, 35 minutes of ischaemia followed by 120 minutes of reperfusion with the administration of 2'-MeCCPA (10nM) in the presence and absence of Wortmannin (100nM) at 30 minutes post reperfusion.

During the ischaemic period, all treatment groups at time matched points significantly decreased the LVDP compared to the normoxic heart group ($p<0.05$) (Figure 5.11). Throughout the period of reperfusion, there was an overall decline in LVDP from all treatment groups however no significance was shown between different treatment groups at time matched points ($p>0.05$) (Figure 5.11).

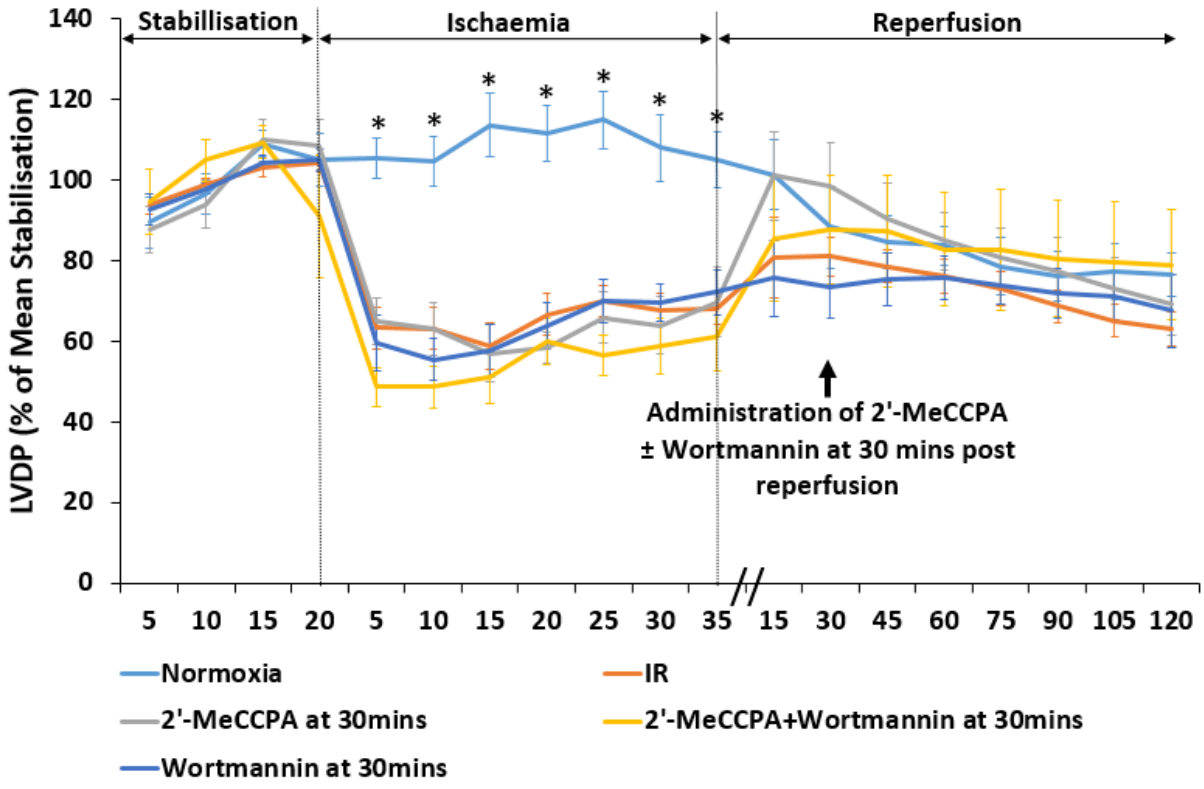


Figure 5. 11 Assessing the effects of 2'-MeCCPA (10nM) in the presence and absence of Wortmannin (100nM) when administered at 30 minutes post reperfusion on left ventricular developed pressure (LVDP) within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Data was presented as Mean±SEM, n=6-8. * $p<0.05$ All groups vs. Normoxia.

There was no significant effects detected on heart rate between all time-matched treatment groups ($p>0.05$) (Figure 5.12).

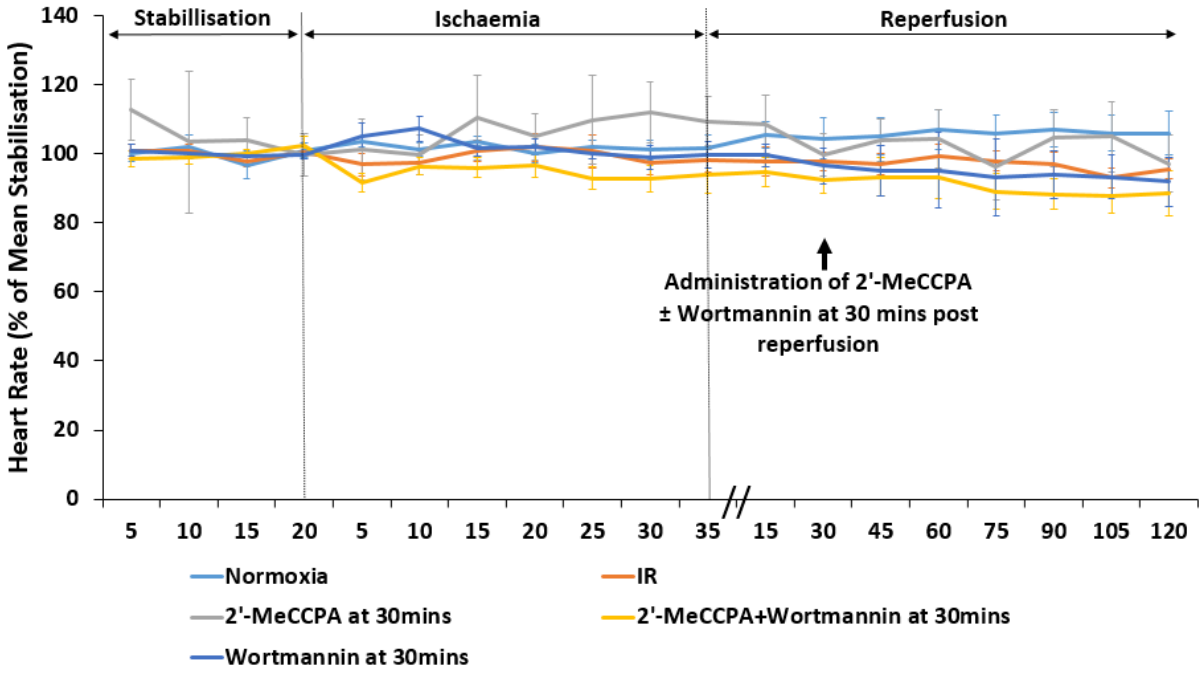


Figure 5. 12 Effects of 2'-MeCCPA (10nM) in the presence and absence of Wortmannin (100nM) when administered at 30 minutes post reperfusion on heart rate within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Data was presented as Mean±SEM, n=6-8.

A significant decrease in coronary flow was observed in all treatment groups within the ischaemic period compared to the normoxic group ($p<0.05$) (Figure 5.13). No significant changes were observed between the treatment groups at each time matched points within the reperfusion period ($p>0.05$) (Figure 5.13).

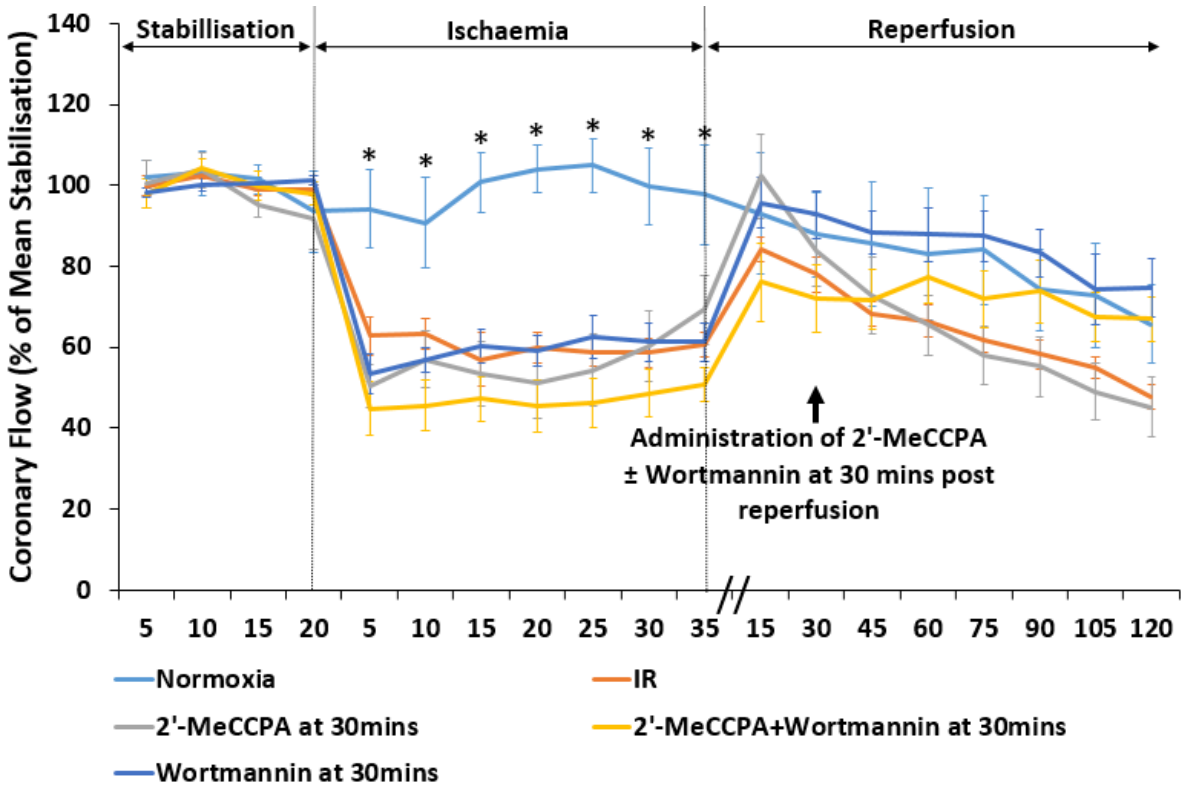


Figure 5. 13 Effects of 2'-MeCCPA (10nM) in the presence and absence of Wortmannin (100nM) when administered at 30 minutes post reperfusion on coronary flow within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Data was presented as Mean±SEM, n=6-8. * $p<0.05$ All groups vs. Normoxia.

5.3.2.2a The Effects of postponing the administration of 2'-MeCCPA (10nM) to 15 minutes post-reperfusion in the presence and absence of PI3K-AKT inhibitor, Wortmannin (100nM) on infarct size to risk ratio (%) in isolated hearts subjected to ischaemia reperfusion injury

Postponement of the administration of A_1 agonist 2'-MeCCPA (10nM) to 15 minutes after the onset of reperfusion was also able to significantly protect the ischaemic-reperfused myocardium from injury. In order to elucidate the intracellular signalling pathway involved to cause this protection, hearts were perfused with 2'-MeCCPA (10nM) in the presence and absence of PI3K-AKT inhibitor Wortmannin (100nM) 15 minutes post reperfusion.

It was found that the administration of the A_1 agonist 2'-MeCCPA (10nM) in the presence of the PI3K-AKT inhibitor, Wortmannin (100nM), administered at 15 minutes into the onset of reperfusion significantly abolished the protection observed when 2'-MeCCPA (10nM) was administered alone at 15 minutes post reperfusion (47$\pm$2% 2'-MeCCPA+Wortmannin@ 15mins Post-R vs. 30$\pm$10% 2'-MeCCPA@15mins Post-R, $p<0.05$) (Figure 5.14).

When Wortmannin (100nM) was administered alone at 15 minutes post reperfusion, there was no significant effect upon the infarct size to risk ratio when compared to the IR control (56$\pm$8% Wortmannin@15mins Post-R vs. 55$\pm$6% IR Control, $p>0.05$) (Figure 5.14).

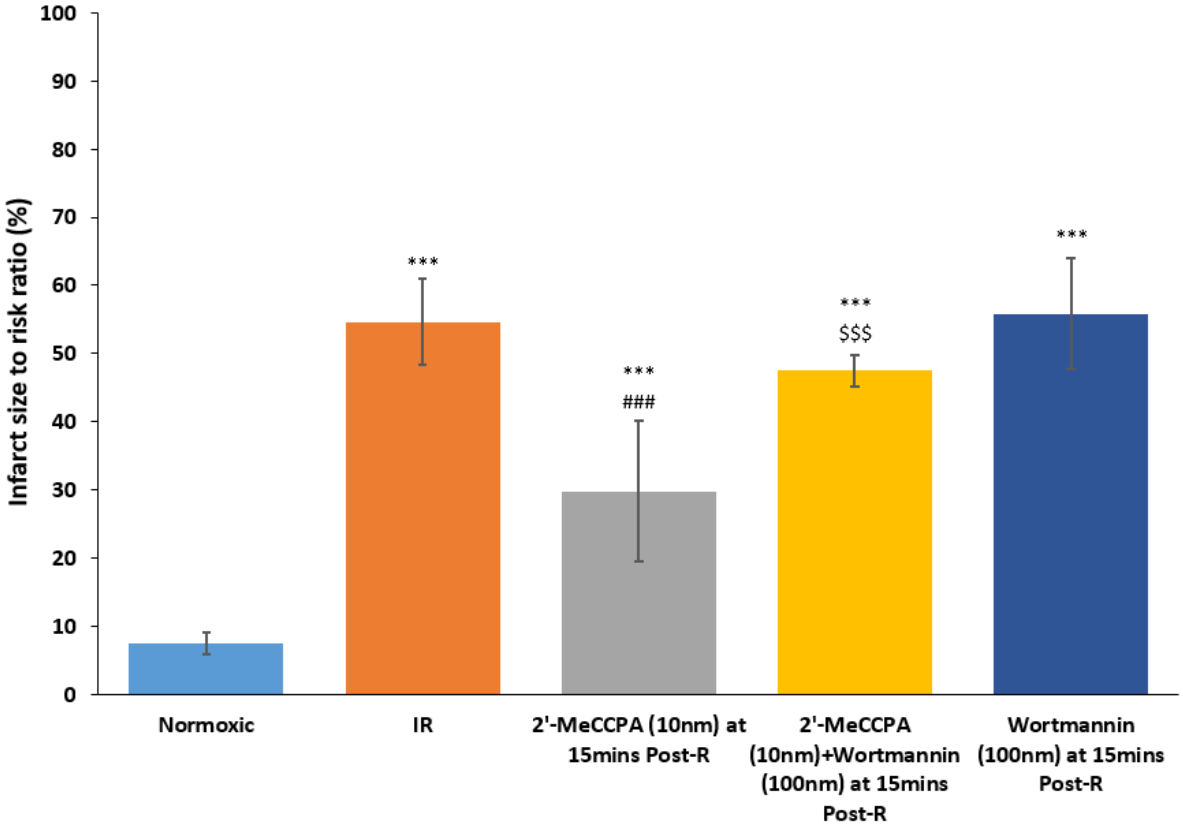


Figure 5. 14 The infarct size to risk ratio (%) in non-treated IR control and 2'-MeCCPA (10nM) treated ischaemic reperfused hearts in the presence and absence of Wortmannin (100nM). Isolated perfused rats' hearts were subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and a further 120 minutes of reperfusion where A_1 adenosine receptor agonist, 2'-MeCCPA (10nM) was administered at 15 minutes post reperfusion in the presence and absence of Wortmannin (100nM). n=6-8 Mean±SEM. *** p<0.001 vs. Normoxia. ### p<0.001 vs. IR control. $$$ p<0.001 vs. 2'-MeCCPA at 15mins Post-R.

5.3.2.2b The Effects of postponing the administration of 2'-MeCCPA (10nM) to 30 minutes post-reperfusion in the presence and absence of PI3K-AKT inhibitor, Wortmannin (100nM) on infarct size to risk ratio (%) within isolated hearts subjected to ischaemia reperfusion injury.

When administration of A_1 adenosine agonist, 2'-MeCCPA (10nM) was postponed to 30 minutes after the onset of reperfusion, significant protection to the myocardium was observed (Figure 3.10). In order to determine whether the PI3K-AKT cell survival pathway contributed towards this protection, hearts were perfused with 2'-MeCCPA (10nM) in the presence and absence of PI3K-AKT inhibitor, Wortmannin (100nM) at 30 minutes post reperfusion.

It was observed that the administration of 2'-MeCCPA (10nM) in the presence of PI3K-AKT inhibitor Wortmannin (100nM) at 30 minutes post reperfusion significantly abolished the protection compared to when 2'-MeCCPA (10nM) was administered alone at 30 minutes post reperfusion (54±3% 2'-MeCCPA+Wortmannin at 30mins Post-R vs. 35±6% 2'-MeCCPA at 30mins Post-R, $p<0.01$) (Figure 5.15).

When Wortmannin (100nM) was administered alone at 30 minutes post reperfusion, no significant effect was observed upon the infarct size to risk ratio in comparison to the IR control (58±7% Wortmannin at 30mins Post-R vs. 55±6% IR control, $p>0.05$) (Figure 5.15).

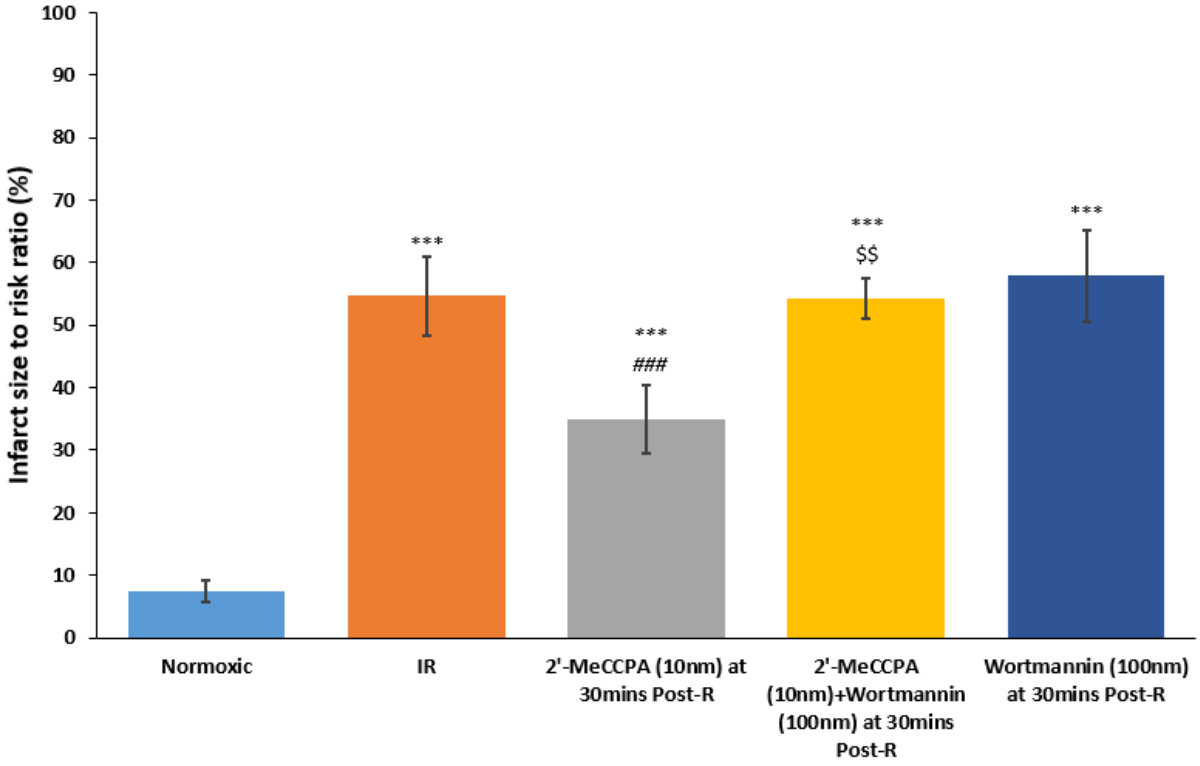


Figure 5. 15 Infarct size to risk ratio (%) in non-treated IR control and 2'-MeCCPA (10nM) treated ischaemic reperfused hearts in the presence and absence of Wortmannin (100nM). Isolated perfused rats' hearts were subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and a further 120 minutes of reperfusion where A_1 adenosine receptor agonist, 2'-MeCCPA (10nM) was administered at 30 minutes post reperfusion in the presence and absence of Wortmannin (100nM). n=6-8 Mean±SEM. *** $p<0.001$ vs. Normoxia. ### $p<0.001$ vs. IR control. $$ $p<0.01$ vs. 2'-MeCCPA at 30mins Post-R.

5.3.2.3a Effects of postponing the administration of 2'-MeCCPA to 15 minutes post reoxygenation on isolated rat cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation from reoxygenation injury via the recruitment of the PI3K-AKT cell signalling pathway and its effects on cellular necrosis and apoptosis.

In order to further determine the cardioprotective role of A_1 adenosine receptor agonist, 2'-MeCCPA (10nM), it was administered 15 minutes post reperfusion to isolated adult rat cardiomyocytes that were subjected to 1 hour of hypoxia and a further 3 hours of reoxygenation. 2'-MeCCPA (10nM) was introduced to the cardiomyocytes 15 minutes after the initiation of reoxygenation in the presence and absence of PI3K inhibitor Wortmannin (100nM).

When the administration of 2'-MeCCPA (10nM) was postponed to 15 minutes post reoxygenation, there was a decrease in the number of apoptotic cardiomyocytes compared to the Hyp/Reox control group (14±5% 2'-MeCCPA at 15mins Post-R vs. 34±6% Hyp/Reox, $p<0.001$) (Figure 5.16).

Postponing the administration of 2'-MeCCPA (10nM) to 15 minutes post reoxygenation significantly decreased the number of necrotic cardiomyocytes when compared to the Hyp/Reox control group (16±4% 2'-MeCCPA at 15mins Post-R vs. 28±7% Hyp/Reox, $p<0.001$) (Figure 5.17).

The administration of 2'-MeCCPA (10nM) at 15 minutes post reperfusion in the presence of PI3K inhibitor Wortmannin (100nM) was able to significantly abolish the anti-apoptotic effects that had been observed when 2'-MeCCPA (10nM) was administered alone at 15 minutes post reperfusion (28±6% 2'-MeCCPA+Wortmannin at 15mins Post-R vs. 14±5% 2'-MeCCPA at 15mins Post-R, $p<0.001$) (Figure 5.16). Moreover, the anti-necrotic effect observed when 2'-MeCCPA (10nM) was administered alone at 15 minutes post reoxygenation was abolished when in the presence of PI3K inhibitor, Wortmannin (100nM) (27±6% 2'-MeCCPA+Wortmannin at 15mins Post-R vs. 2'-MeCCPA at 15mins Post-R, $p<0.001$) (Figure 5.17).

The administration of Wortmannin (100nM) alone at 15 minutes post reperfusion had no significant effect upon cellular apoptosis on cardiomyocytes when compared to the Hyp/Reox

group (27±5% Wortmannin at 15mins Post-R vs. 34±6% Hyp/Reox, p>0.05) (Figure 5.16). Administration of Wortmannin (100nM) alone at 15 minutes into reoxygenation had no significant effect on cardiomyocyte necrosis when compared to the Hyp/Reox group (25±6% Wortmannin at 15mins Post-R vs. 28±7% Hyp/Reox, p>0.05) (Figure 5.17).

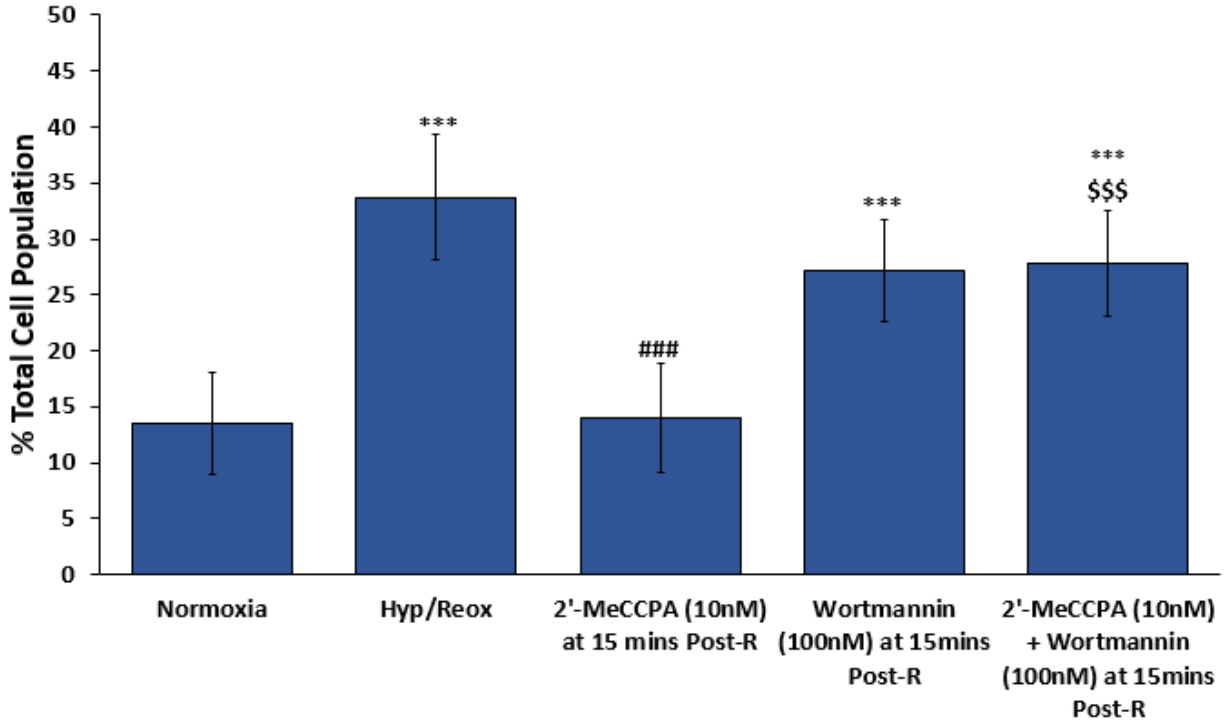


Figure 5. 16 Assessment of apoptosis in isolated rat cardiomyocytes subjected to 4 hours of oxygenation (Normoxia) or 1 hours of hypoxia and 3 hours of reoxygenation. A_1AR agonist 2'-MeCCPA (10nM) was administered in the presence and absence of PI3K inhibitor Wortmannin (100nM) at 15 minutes post reoxygenation in order to assess the involvement of the PI3K-AKT cell signalling pathway. Results are shown at Mean±SEM and expressed as a percentage of 10 000 cells counted from 6 individual experiments. *** p<0.001 vs. Normoxia. ### p<0.001 vs. Hyp/Reox. $$$ p<0.001 vs. 2'-MeCCPA at 15mins Post-R.

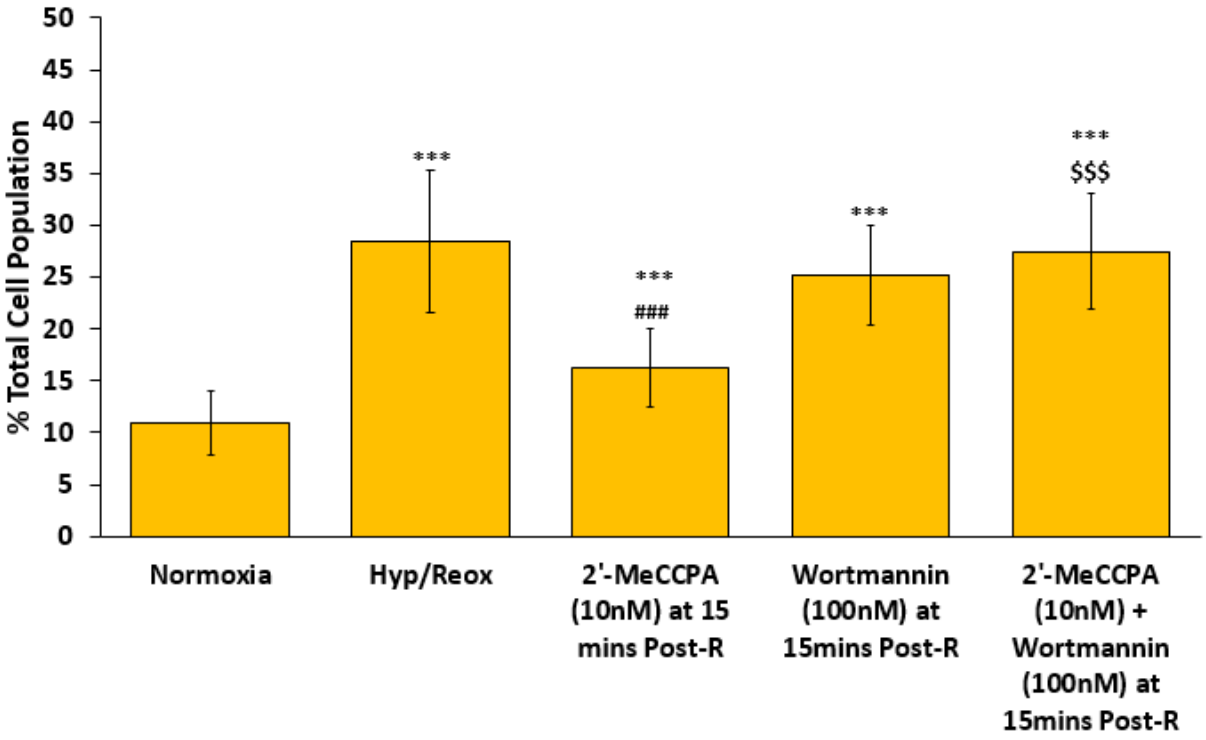


Figure 5. 17 Assessment of necrosis in isolated rat cardiomyocytes subjected to 4 hours of oxygenation (Normoxia) or 1 hours of hypoxia and 3 hours of reoxygenation. A_1AR agonist 2'-MeCCPA (10nM) was administered in the presence and absence of PI3K inhibitor Wortmannin (100nM) at 15 minutes post reoxygenation in order to assess the involvement of the PI3K-AKT cell signalling pathway. Results are shown at Mean±SEM and expressed as a percentage of 10 000 cells counted from 6 individual experiments. *** p<0.001 vs. Normoxia. ### p<0.001 vs. Hyp/Reox. $$$ p<0.001 vs. 2'-MeCCPA at 15mins Post-R.

5.3.2.3b Effects of postponing the administration of 2'-MeCCPA to 30 minutes post reoxygenation on isolated rat cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation from reoxygenation injury via the recruitment of the PI3K-AKT cell signalling pathway and its effects on cellular necrosis and apoptosis.

It was observed that when postponing the administration of A_1 adenosine receptor agonist 2'-MeCCPA (10nM) to 30 minutes post reoxygenation, a significant decrease in cellular apoptosis was observed in comparison to the non-treated Hyp/Reox group (15±5% 2'-MeCCPA at 30mins Post-R vs. 34±6% Hyp/Reox, p<0.001) (Figure 5.18). The postponement of 2'-MeCCPA (10nM) to 30 minutes post reoxygenation was also able to significantly decrease cellular necrosis compared to the Hyp/Reox group (17±3% 2'-MeCCPA at 30mins Post-R vs. 28±7% Hyp/Reox, p<0.001) (Figure 5.19).

The administration of 2'-MeCCPA (10nM) at 30 minutes post reperfusion was able to significantly decrease cellular apoptosis however when 2'-MeCCPA (10nM) and Wortmannin (100nM) were administered in conjunction, the decrease in cellular apoptosis was abolished (15±5% 2'-MeCCPA at 30mins Post-R vs. 27±4% 2'-MeCCPA+Wortmannin at 30mins Post-R, $p<0.001$) (Figure 5.18). Furthermore, it was observed that the anti-necrotic effects of when 2'-MeCCPA (10nM) was administered alone at 30 minutes post reoxygenation were abolished in the presence of PI3K inhibitor Wortmannin (100nM) (17±3% 2'-MeCCPA at 30mins Post-R vs. 27±5% 2'-MeCCPA+Wortmannin 30mins Post-R, $p<0.001$) (Figure 5.19).

Administration of Wortmannin (100nM) alone at 30 minutes post reoxygenation had no significant effect on cardiomyocyte apoptosis when compared to the Hyp/Reox control group (24±5% Wortmannin at 30mins Post-R vs. 35±6% Hyp/Reox, $p>0.05$) (Figure 5.18). The administration of Wortmannin (100nM) alone at 30 minutes post reperfusion had no significant effect on cardiomyocyte necrosis when compared to the Hyp/Reox control group (28±5% Wortmannin at 30mins Post-R vs. 28±7% Hyp/Reox, $p>0.05$) (Figure 5.19).

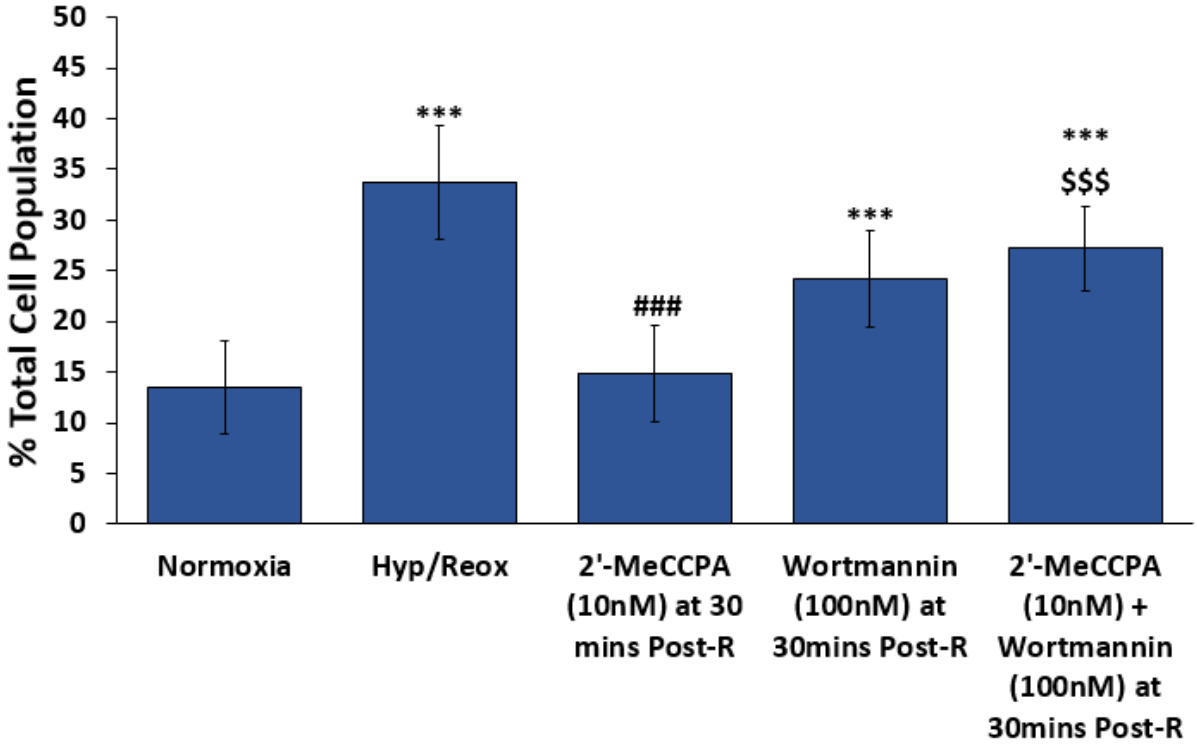


Figure 5. 18 Assessment of apoptosis in isolated rat cardiomyocytes subjected to 4 hours of oxygenation (Normoxia) or 1 hours of hypoxia and 3 hours of reoxygenation. A_1AR agonist 2'-MeCCPA (10nM) was administered in the presence and absence of PI3K inhibitor Wortmannin (100nM) at 30 minutes post reoxygenation in order to assess the involvement of the PI3K-AKT cell signalling pathway. Results are shown at Mean±SEM and expressed as a percentage of 10 000 cells counted from 6 individual experiments. *** $p<0.001$ vs. Normoxia. ### $p<0.001$ vs. Hyp/Reox. $$$ $p<0.001$ vs. 2'-MeCCPA at 30mins Post-R.

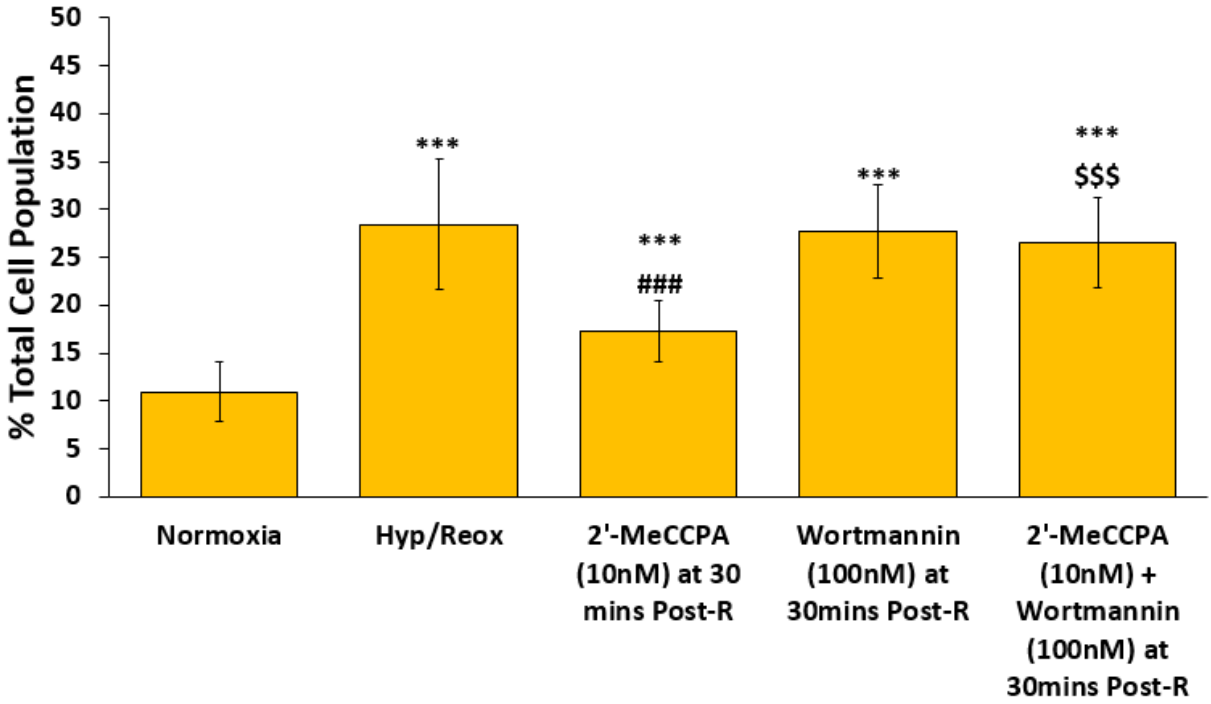


Figure 5. 19 Assessment of necrosis in isolated rat cardiomyocytes subjected to 4 hours of oxygenation (Normoxia) or 1 hours of hypoxia and 3 hours of reoxygenation. A_1AR agonist 2'-MeCCPA (10nM) was administered in the presence and absence of PI3K inhibitor Wortmannin (100nM) at 30 minutes post reoxygenation in order to assess the involvement of the PI3K-AKT cell signalling pathway. Results are shown at Mean±SEM and expressed as a percentage of 10 000 cells counted from 6 individual experiments. *** p<0.001 vs. Normoxia. ### p<0.001 vs. Hyp/Reox. $$$ p<0.001 vs. 2'-MeCCPA at 30mins Post-R.

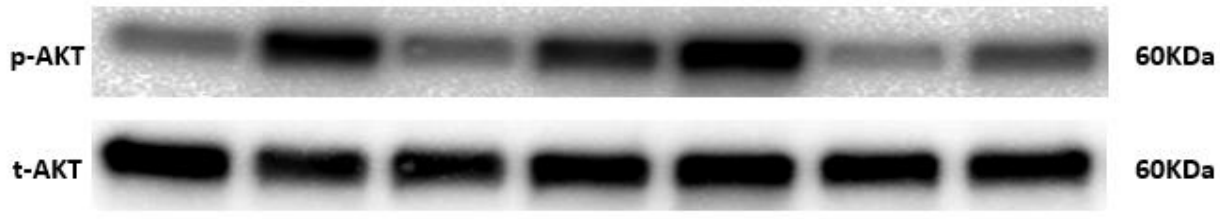
5.3.2.4a The role of p-AKT $_{(ser473)}$ in 2'-MeCCPA mediated cardioprotection when administered at 15 minutes post reperfusion.

Having been able to determine that 2'-MeCCPA (10nM) protects isolated perfused rat heart from ischaemia reperfusion injury when administered at 15 minutes post reperfusion via the PI3K-AKT cell signalling pathway This meant it was important to further examine the effects of the A_1 adenosine agonist and the PI3K inhibitor Wortmannin (100nM) on $AKT_{(ser473)}$ phosphorylation. Hearts were harvested 25 and 35 minutes after reperfusion where A_1 adenosine agonist, 2'-MeCCPA (10nM) was administered at 15 minutes post reperfusion.

Within the control hearts, $AKT_{(ser473)}$ phosphorylation was observed at 25 and 35 minutes of reperfusion (Figure 5.20). It was observed that the administration of 2'-MeCCPA (10nM) significantly upregulated the phosphorylation of $AKT_{(ser473)}$ with maximal phosphorylation of $AKT_{(ser473)}$ at 25 minutes of reperfusion compared with the time matched non-treated control

where the A_1 agonist 2'-MeCCPA (10nM) was perfused at 15 minutes post reperfusion ($p<0.001$) (Figure 5.20 and Figure 5.21).

The administration of 2'-MeCCPA (10nM) at 15 minutes post reperfusion significantly upregulated AKT $_{(ser473)}$ phosphorylation after 25 minutes of reperfusion; this was then abolished in the presence of PI3K inhibitor Wortmannin (100nM) ($p<0.001$) (Figure 5.21). The administration of the PI3K inhibitor Wortmannin (100nM) alone at 15 minutes post reperfusion until a total of 25 minutes of reperfusion caused a significant decrease in AKT$_{(ser473)}$ phosphorylation when compared to the time matched non-treated control group ($p<0.01$) (Figure 5.20 and Figure 5.21).



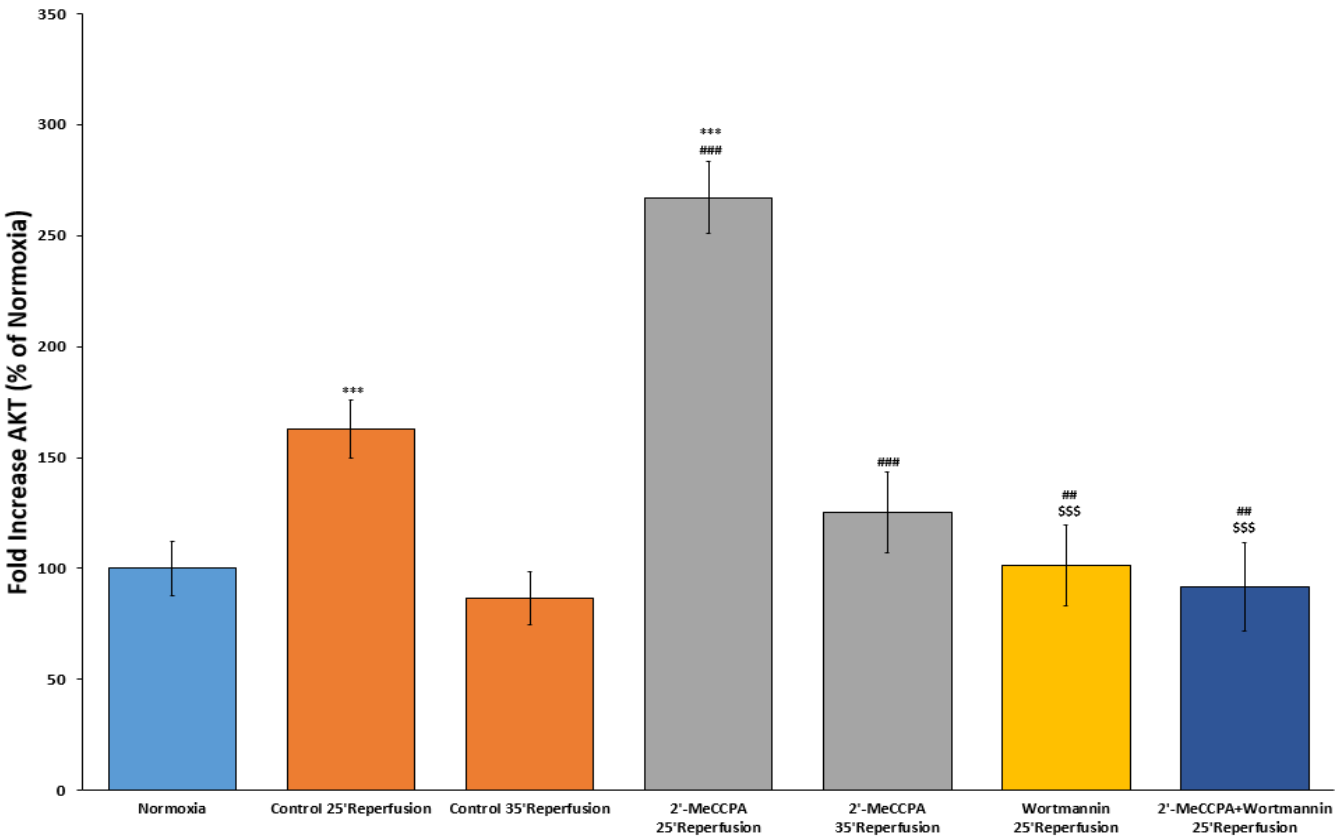


Figure 5. 20 The assessment of AKT$_{(ser473)}$ phosphorylation in isolated hearts that were subjected to 60 minutes of perfusion (Normoxia) or 20 minutes of stabilisation, 35 minutes of ischaemia followed by 25 or 35 minutes of reperfusion in the presence and absence of A_1 adenosine receptor agonist, 2'-MeCCPA (10nM) administered at 15 minutes post-reperfusion. 2'-MeCCPA (10nM) was administered in the presence and absence of PI3K inhibitor Wortmannin (100nM) at 15 minutes post-reperfusion. Results were shown as Mean ± SEM of four experiments. ### p<0.001 2'-MeCCPA 25', 35' vs. Control 25', 35' respectively. $$$ p<0.001 2'-MeCCPA+Wortmannin 25', Wortmannin 25' vs. 2'-MeCCPA 25'. *** p<0.001 Control 25', 2'-MeCCPA 25' vs. Normoxia.

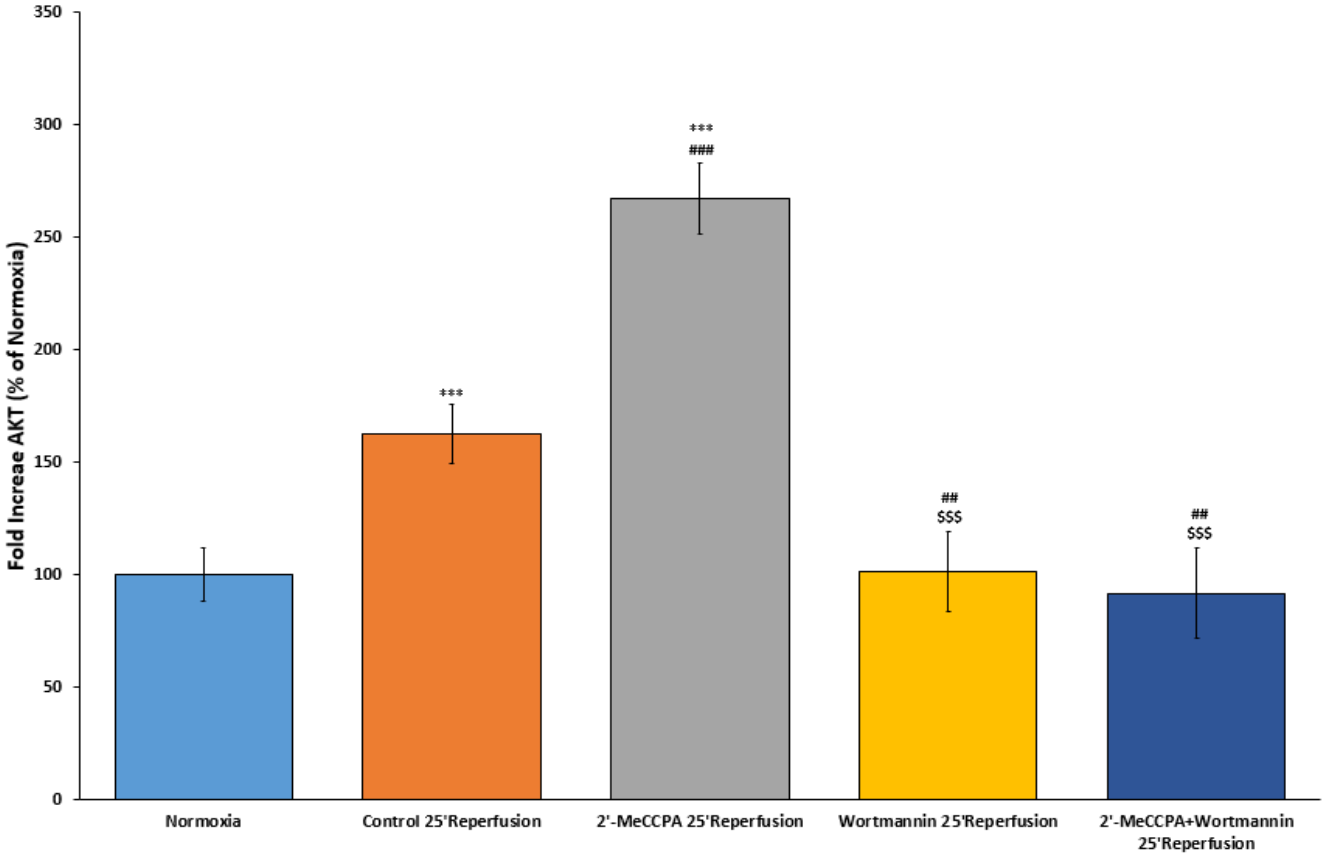


Figure 5. 21 The comparison of AKT $_{(ser473)}$ phosphorylation in isolated hearts subjected to 20 minutes stabilisation, 35 minutes of ischaemia followed by 25 minutes of reperfusion. A_1 adenosine receptor agonist, 2'-MeCCPA (10nM) was administered at 15 minutes post reperfusion in the presence and absence of PI3K inhibitor Wortmannin (100nM). Results are shown as Mean±SEM of 4 experiments. *** p<0.001 vs. Normoxia. ### p<0.001 vs. Control 25'Reperfusion. ## p<0.01 vs. Control 25'Reperfusion. $$$ p<0.001 vs. 2'-MeCCPA 25'Reperfusion.

5.3.2.4b The role of AKT phosphorylation within the PI3K-AKT signalling pathway in 2'-MeCCPA mediated cardioprotection when administered at 30 minutes post reperfusion.

It was previously shown in Chapter 3 that when 2'-MeCCPA (10nM) was administered at 30 minutes post reperfusion, development of infarction was limited within ischaemic reperfused hearts. This protection was then blocked in the presence of the PI3K inhibitor Wortmannin (100nM).

In order to determine whether the protection afforded by 2'-MeCCPA (10nM) when administered at 30 minutes post reperfusion is via the recruitment of the PI3K-AKT cell survival pathway, heart tissues were treated with 2'-MeCCPA (10nM) in the presence and absence of the PI3K inhibitor Wortmannin (100nM) at 30 minutes post reperfusion and $AKT_{(ser473)}$ phosphorylation was assessed.

Within the control heart groups, $AKT_{(ser473)}$ phosphorylation was observed at 40 and 50 minutes of reperfusion. It was observed that the administration of 2'-MeCCPA (10nM) specifically at 30 minutes post reperfusion significantly upregulated the phosphorylation of $AKT_{(ser473)}$ after 40 minutes of reperfusion compared to its time matched control ($p<0.001$) (Figure 5.22). The administration of 2'-MeCCPA (10nM) and Wortmannin (100nM) together at 30 minutes post reperfusion significantly abolished the increase in $AKT_{(ser473)}$ phosphorylation incurred by 2'-MeCCPA (10nM) alone after 40 minutes of reperfusion ($p<0.001$) (Figure 5.22 and Figure 5.23).

The administration of 2'-MeCCPA (10nM) at 30 minutes post reperfusion showed a significant down regulation of $AKT_{(ser473)}$ phosphorylation after 50 minutes of reperfusion compared to its time matched control ($p<0.001$) (Figure 5.22).

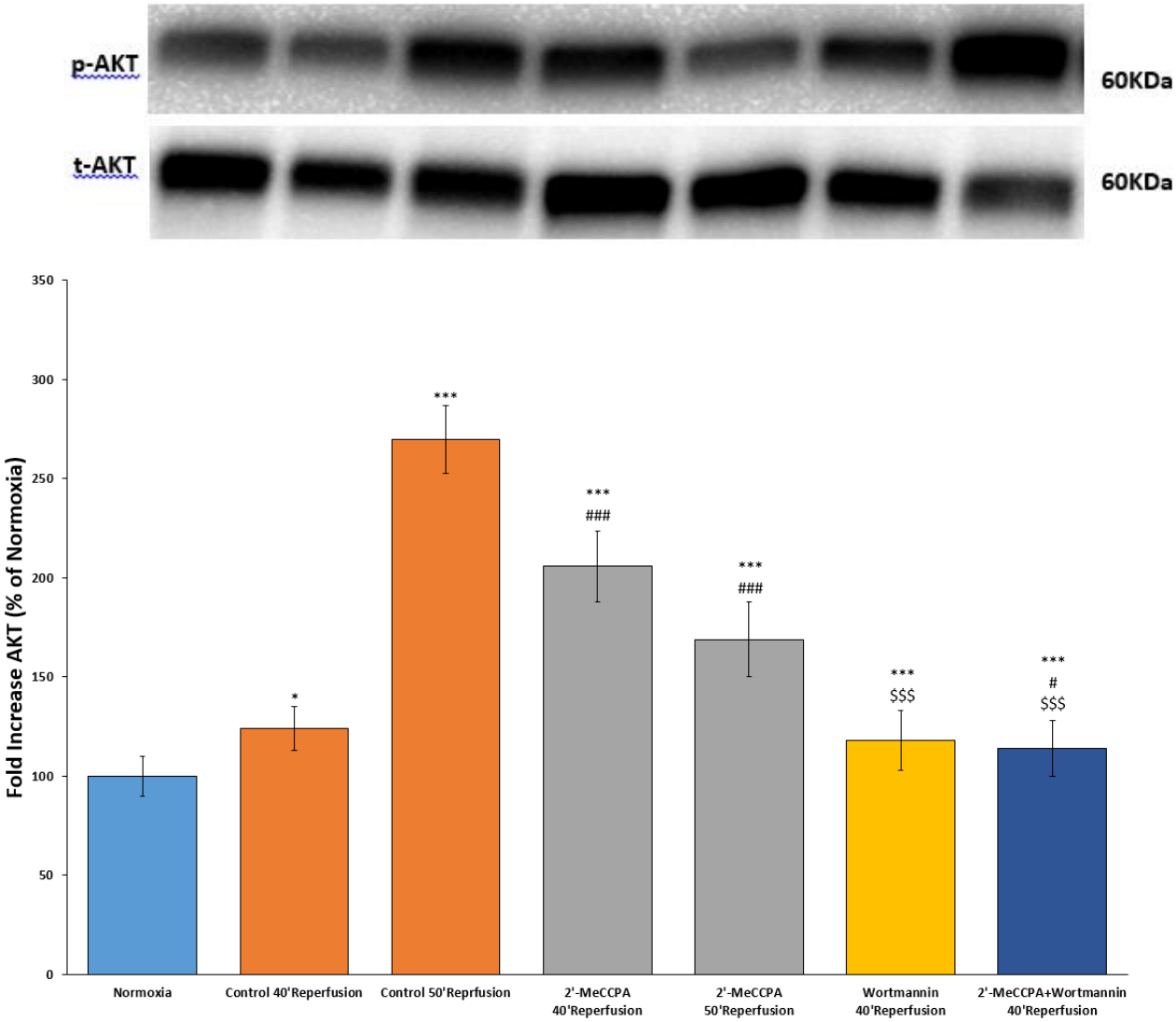


Figure 5. 22 The assessment of p-AKT phosphorylation in isolated hearts subjected to 60 minutes of perfusion (Normoxia) or 20 minutes of stabilisation, 35 minutes of ischaemia followed by 40 or 50 minutes of reperfusion in the presence and absence of A_1 adenosine receptor agonist 2'-MeCCPA (10nM). The PI3K inhibitor Wortmannin (100nM) was administered at reperfusion in the presence and absence of 2'-MeCCPA (10nM). Results are shown as Mean ± SEM of four experiments. *** p<0.001 Control 50', 2'-MeCCPA 40' and 50', Wortmannin 40', 2'-MeCCPA+Wortmannin 40' vs. Normoxia. * p<0.05 Control 40' vs. Normoxia. ### p<0.001 2'-MeCCPA 40' and 50' vs. Control 40' and 50' respectively. $$$ p<0.001 Wortmannin 40' vs. 2'-MeCCPA 40'. $$ 0.01 2'-MeCCPA+Wortmannin 40' vs. 2'-MeCCPA 40'.

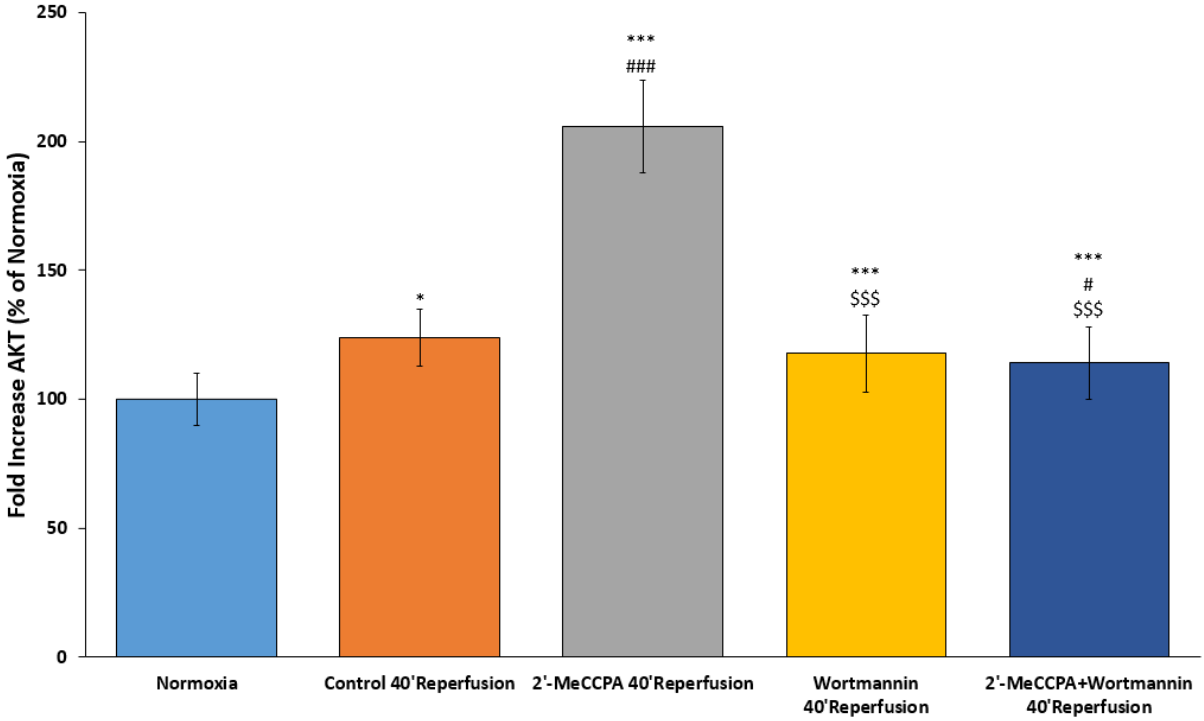


Figure 5. 23 A comparison of AKT phosphorylation within isolated rat hearts subjected to 60 minutes of perfusion (Normoxia) or 20 minutes of stabilisation, 35 minutes of ischaemia followed by 40 minutes of reperfusion in the presence and absence of A_1 adenosine receptor agonist (10nM). The PI3K inhibitor Wortmannin (100nM) was administered in the presence and absence of 2'-MeCCPA (10nM). Results are shown as Mean ± SEM of four experiments. * p<0.05 vs. Normoxia. *** p<0.001 vs. Normoxia. ### p<0.001 vs. Control 40'. # p<0.05 vs. Control 40'. $$$ p<0.001 vs. 2'-MeCCPA 40'.

5.3.2.5a The effect of 2'-MeCCPA (10nM) when administered at 15 minutes post reoxygenation on cleaved-caspase 3 activity in isolated adult rat cardiomyocytes.

In Chapter 3 the A_1 adenosine receptor agonist 2'-MeCCPA (1nM, 10nM, 100nM and 1µM) significantly decreased cleaved-caspase 3 activity when administered at the onset of reoxygenation. Chapter 3 also stated that when 2'-MeCCPA (10nM) was administered at 15 minutes post-reoxygenation, cardioprotection was conferred via decreasing the activity of cleaved-caspase 3 within isolated adult rat cardiomyocytes that were then subjected to 1 hour of hypoxia and 3 hours of reoxygenation. Moreover, to determine whether the protection was caused via the recruitment of the PI3K-AKT cell signalling pathway, 2'-MeCCPA (10nM) was administered at 15 minutes post reoxygenation in the presence and absence of PI3K inhibitor Wortmannin (100nM).

The administration of 2'-MeCCPA (10nM) at 15 minutes post reoxygenation significantly decreased cleaved-caspase 3 activity compared to the non-treated Hyp/Reox group (232 ± 16% 2'-MeCCPA 15mins Post-R vs. 313 ± 35% Hyp/Reox, $p<0.01$) (Figure 5.24).

Administration of 2'-MeCCPA (10nM) significantly decreased cleaved-caspase 3 activity when administered 15 minutes post reperfusion however this was decrease was significantly abolished in the presence of PI3K inhibitor Wortmannin (100nM) (232 ± 16% 2'-MeCCPA 15mins Post-R vs. 321 ± 22% 2'-MeCCPA+Wortmannin 15mins Post-R, $p<0.001$) (Figure 5.24).

The administration of Wortmannin (100nM) alone at 15 minutes post reoxygenation resulted in a decrease in cleaved-caspase 3 activity when compared to the Hyp/Reox control group however this was not a significant change (285 ± 34% Wortmannin 15mins Post-R vs. 313 ± 35% Hyp/Reox, $p>0.05$) (Figure 5.24).

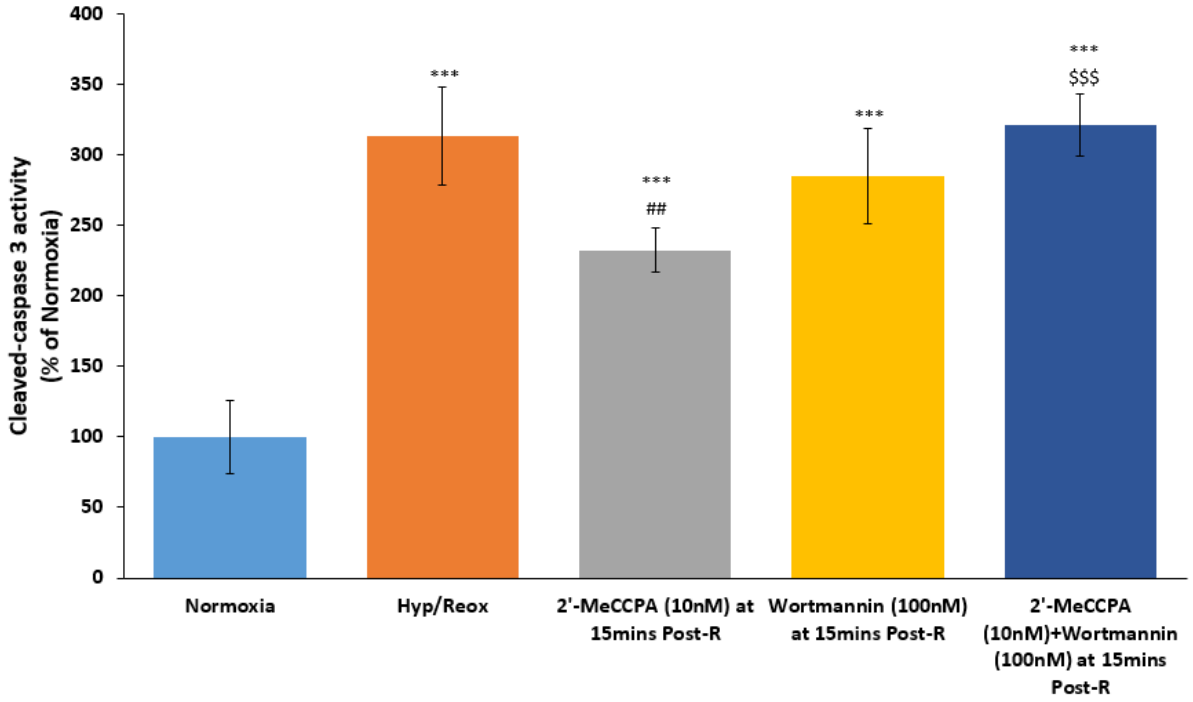


Figure 5. 24 Cleaved-caspase 3 activity within isolated adult rat cardiomyocytes subjected to 4 hours of oxygenation (Normoxia) or 1 hour of hypoxia followed by 3 hours of reoxygenation (Hyp/Reox). The A_1 agonist 2'-MeCCPA (10nM) was administered 15 minutes post reoxygenation in the presence and absence of the PI3K inhibitor Wortmannin (100nM). Mean±SEM of 6 individual experiments. *** $p<0.001$ vs. Normoxia. ## $p<0.01$ vs. Hyp/Reox. $$$ $p<0.001$ vs. 2'-MeCCPA 15mins Post-R.

5.3.2.5b The effect of 2'-MeCCPA (10nM) when administered at 30 minutes post reoxygenation on cleaved caspase-3 activity in isolated adult rat cardiomyocytes.

The administration of 2'-MeCCPA (10nM) at 30 minutes post reoxygenation decreased cleaved-caspase 3 activity in isolated adult rat cardiomyocytes subjected to 1 hours of hypoxia and 3 hours of reoxygenation compared with the Hyp/Reox control group, however this was not a significant change (263±27% 2'-MeCCPA 30mins Post-R vs. 313±35% Hyp/Reox, $p>0.05$) (Figure 5.25).

In order to determine whether the non-significant decrease in cleaved-caspase 3 by 2'-MeCCPA (10nM) at 30 minutes post reoxygenation was via PI3K-AKT cell signalling pathway, the PI3K inhibitor Wortmannin (100nM) was used. The administration of 2'-MeCCPA (10nM) at 30 minutes post reoxygenation in the presence of PI3K inhibitor Wortmannin (100nM) significantly abolished the decrease observed in cleaved-caspase 3 when 2'-MeCCPA (10nM) was administered alone at 30 minutes post reoxygenation (371±22% 2'-MeCCPA+Wortmannin 30mins Post-R vs. 263±27% 2'-MeCCPA 30mins Post R, $p<0.05$) (Figure 5.25).

The administration of Wortmannin (100nM) alone at 30 minutes post reoxygenation resulted in a decrease in cleaved-caspase 3 activity compared to the Hyp/Reox control group however this was not a significant decrease (301±40% Wortmannin 30mins Post-R vs. 313±35% Hyp/Reox, $p>0.05$) (Figure 5.25).

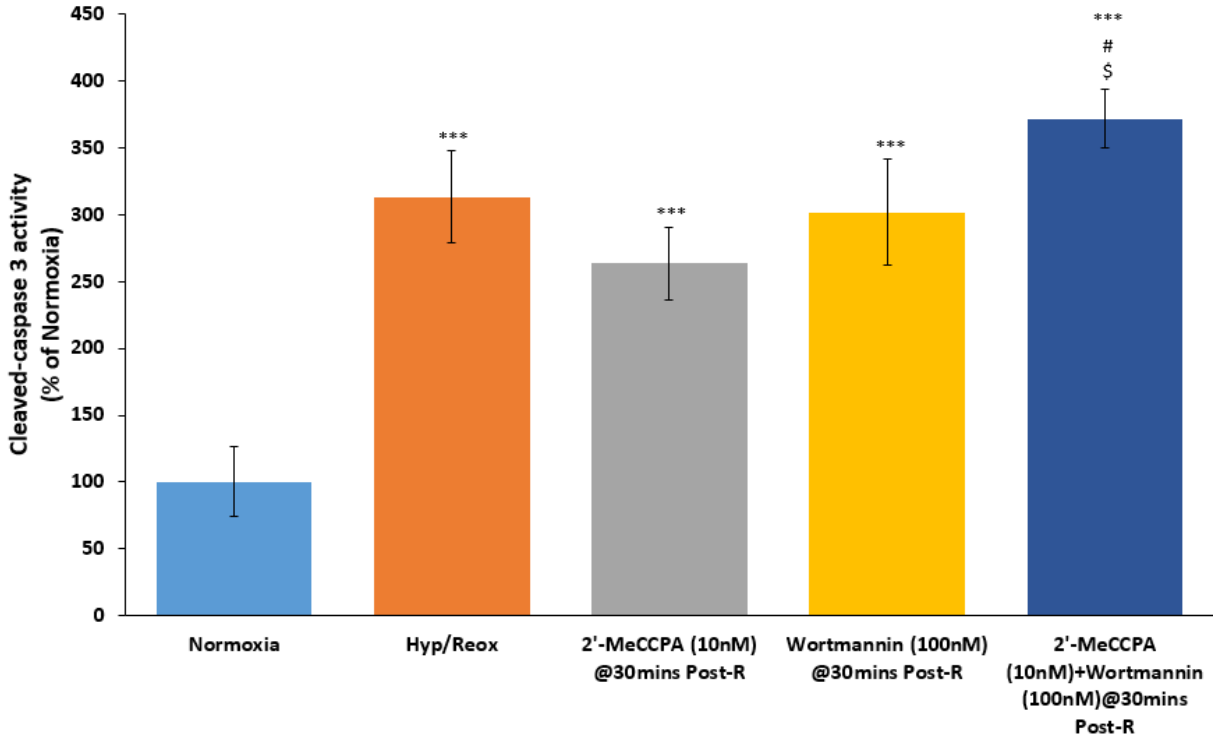


Figure 5. 25 Cleaved-caspase 3 activity within isolated adult rat cardiomyocytes subjected to 4 hours of oxygenation (Normoxia) or 1 hour of hypoxia followed by 3 hours of reoxygenation (Hyp/Reox). The A_1 agonist 2'-MeCCPA (10nM) was administered 30 minutes post reoxygenation in the presence and absence of the PI3K inhibitor Wortmannin (100nM). Mean±SEM of 6 individual experiments. *** $p<0.001$ vs. Normoxia. # $p<0.05$ vs. Hyp/Reox. $ $p<0.05$ vs. 2'-MeCCPA 30mins Post-R.

5.4 Discussion

The PI3K-AKT cell signalling pathway has been a very well-studied pro-survival pathway in reference to its role in ischaemia reperfusion injury. AKT is the downstream target of PI3K and it can in turn activate a number of those downstream targets. These targets include eNOS, p70S6K, BAD and PKC (Cross et al. 2000; Park et al. 2006). The exact cellular mechanisms that are involved within the cardioprotection that is ensured via A_1 adenosine receptors in the ischaemic reperfused myocardium still remains unclear as several studies have shown to activate different pathways in response to A_1 adenosine receptor activation.

Previous research conducted by Kudo et al. (2002) demonstrated that pharmacological preconditioning in male mice with A_1AR agonist CCPA induced cardioprotection via the activation of Protein Kinase- δ (PKC-δ) in a way that it was able to reduce infarct size (Kudo et al. 2002). Taking this into consideration and research into literature so far, the activation of PKC-δ seems to have occurred through the phenomenon of pharmacological preconditioning. Our study applied the protocol of post-reperfusion activation of A_1 adenosine receptors after the onset of reperfusion and found that the PI3K-AKT cell signalling pathway played a major role towards cardioprotection when 2'-MeCCPA was administered at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion. This cardioprotective effect was observed through a reduction in infarct size. Whereas when A_1AR agonist 2'-MeCCPA (10nM) was administered alongside PI3K inhibitor Wortmannin (100nM) at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion, there was an increase in infarct size, therefore implying that the PI3K-AKT cell signalling pathway played a major role in A_1 adenosine mediated cardioprotection.

Although protection, in the form of a reduction of infarct size was observed when 2'-MeCCPA (10nM) was administered at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion, as the different time-points were studied, it was found that protection became less as time went on.

This current study also showed that when the A_1AR agonist 2'-MeCCPA was administered at the onset of reoxygenation, 15 minutes and 30 minutes post-reoxygenation, there was a decrease in cellular apoptosis and necrosis which also meant there was a decrease in caspase-3 activity at all time-points and this decrease was reversed when 2'-MeCCPA (10nM) was

administered alongside PI3K inhibitor Wortmannin (100nM) implying that cardioprotection, in the form of reducing cell death and caspase-3 activity, was implicated through the PI3K-AKT cell signalling pathway. Extensive amounts of research in this area have not been carried out, especially with the recruitment of the PI3K-AKT cell signalling pathway in delayed reperfusion activation of A_1 adenosine receptor cardioprotection; however a previous study conducted by Regan et al. (2003) suggested that the cardioprotection observed in transgenic mice with an overexpression of A_1 adenosine receptors attenuated ischaemia-reperfusion induced apoptosis and caspase-3 but this was independent of the acute PI3K signalling pathway. This confounding data is contradictory to the data observed within our study and could be due to the fact that there was no A_1AR agonist specifically used to target the A_1 adenosine receptors within the study by Regan et al. (2003). Our study also used adult male rats whereas Regan et al. (2003) used transgenic mice which may be the reason for these confounding results. Although hearts had an overexpression of A_1 adenosine receptors, they were not specifically activated by a specific A_1AR agonist to detect the specific effects of the A_1 adenosine receptor in accordance with the PI3K cell signalling pathway whereas our can imply study that the PI3K-AKT cell signalling pathway is activated upon A_1 adenosine receptor activation to cause a decrease in infarct size as well as a reduction in apoptosis and necrosis followed by a decrease in caspase-3 activity.

Further investigation into the links between the PI3K-AKT signalling pathway and targets such as eNOS, p70S6K, BAD and PKC can be made in future investigation to confer if they enhance the level of cardioprotection observed in our study.

Previous research conducted by Wildmann et al. (1998) have shown that the upregulation of caspases can causes degradation of proteins such as AKT and ERK1 throughout cellular injury and when caspase inhibitors are administered they can lead to an overall increase in pro-survival proteins which may be useful in future research and could provide a better understanding of the A_1 adenosine receptor playing a role in the decrease in caspase-3 activity due to the activation of the PI3K-AKT cell signalling pathway.

Our findings expressed that when A_1AR agonist 2'-MeCCPA (10nM) was administered at the onset of reperfusion, there was a significant upregulation of AKT observed compared to when 2'-MeCCPA (10nM) was administered alongside Wortmannin (100nM) and this was also observed when 2'-MeCCPA (10nM) was administered at 15 minutes and 30 minutes post-

reperfusion. This implies that within the first 30 minute window of reperfusion, the A_1 adenosine receptor activation can cause protection to the myocardium after an ischaemic insult through the recruitment of the PI3K-AKT cell signalling pathway. This can be supported by research conducted by Hausenloy and Yellon (2004) who suggested that apoptotic cell death can be reduced during the early phase of reperfusion to reduce lethal reperfusion-induced injury (Hausenloy and Yellon 2004).

AKT has the ability to phosphorylate many downstream targets and previous literature has evidenced the infarct sparing effects of A_3 adenosine receptor agonist IB-MECA in isolated rat heart. This cardioprotective effect through the A_3 adenosine receptor also increased AKT phosphorylation and this protection was lost upon the PI3K inhibition with Wortmannin. With extensive research conducted into the A_3 adenosine receptor, it was only appropriate to investigate these effects upon the activation of the A_1 adenosine receptor. Our study found that AKT phosphorylation increased when A_1AR agonist 2'-MeCCPA (10nM) was administered at the onset of reperfusion as well as being administered 15 minutes and 30 minutes post-reperfusion. This could also imply that the A_3 adenosine receptor activates the PI3K-AKT cell signalling pathway in order to ensure cardioprotection as well as the A_1 adenosine receptor, when activated, recruiting the PI3K-AKT cell signalling pathway alongside to cause cardioprotective effects too.

Our study can imply that when the A_1 adenosine agonist 2'-MeCCPA was administered at 15 minutes post reperfusion/reoxygenation, there was a significant limiting effect upon infarct development in the ischaemic reperfused myocardium and cell death (apoptosis and necrosis) within adult rat cardiomyocytes that were subjected to hypoxia-reoxygenation. This protection can implicate the recruitment of the PI3K-AKT cell signally pathway where 2'-MeCCPA (10nM) also upregulated the phosphorylation of AKT when compared to the control hearts. The critical role of the PI3K-AKT cell signalling pathway for 2'-MeCCPA mediated cardioprotection when administered 15 minutes after reperfusion was determined using the PI3K inhibitor Wortmannin. Wortmannin abolished any cardioprotection previously observed. The A_1 agonist was also seen to decrease the activity of cleaved caspase-3 which was also reversed by Wortmannin (PI3K inhibitor). Postponing the administration of 2'-MeCCPA to 30 minutes post-reperfusion also attenuated myocardial infarction in isolated rat heart in a Wortmannin sensitive manner by the upregulation of AKT, decrease in cell death

(apoptosis and necrosis) and a decrease in cleaved caspase-3 activity. This post-reperfusion/reoxygenation activation of A_1 adenosine receptors has not currently been investigated which is where our findings can be rendered useful.

Previously published literature has shown that when pharmacological agents are administered at the onset of reperfusion, cardioprotection can be ensured via the recruitment of the PI3K-AKT cell signalling pathway. A study by Jonassen et al. (2001) who administered Insulin at the onset of reperfusion to activate the PI3K-AKT cell signalling pathway was able to limit myocardial infarction. It can also be implied from this current study that when 2'-MeCCPA was administered at the onset of reperfusion to limit myocardial infarction via the infarct limiting effects, a decrease in cell death and cleaved caspase-3 activity and an increase in AKT phosphorylation. Therefore, although it has already been published that administration of pharmacological agents at the onset of reperfusion can limit ischaemia-reperfusion injury, the exploration of the activation of the A_1 adenosine receptor to limit ischaemia reperfusion injury at the onset of reperfusion as well as post-reperfusion activation was an area of further research which has been explored within this study.

Bibli and colleagues also investigated that the activation of A_1ARs via the administration of A_1AR agonist CCPA throughout the phenomenon of post-conditioning significantly upregulated AKT levels to confer protection to male rabbit hearts which really consolidated how the PI3K branch of the RISK pathway plays a vital part in the cardioprotection observed within this current study.

Further experiments could be carried out in order to determine the role of cellular crosstalk between the PI3K-AKT cell signalling pathways in accordance to the MEK1/2-ERK1/2 cell signalling pathway.

5.5 Summary of Findings

In summary, this study implies that the A_1 adenosine receptor agonist, 2'-MeCCPA can reduce myocardial ischaemia reperfusion injury in Sprague Dawley rat model via the recruitment of the PI3K-AKT cell survival pathway through the activation of A_1 adenosine receptors at the onset of reperfusion, 15 minutes post reperfusion and 30 minutes post-reperfusion. Our results show that:

- The activation of the A_1 adenosine receptor at the **onset of reperfusion** is suggested to offer cardioprotection via the recruitment of the PI3K-AKT cell signalling pathway. This can be observed through the limiting infarct effects, a decrease in cell death (apoptosis and necrosis), and a decrease in cleaved caspase-3 activity and an increase in p-AKT phosphorylation through western blots. These cardioprotective effects have been seen to be reversed with PI3K inhibitor Wortmannin. This implies that cardioprotection can potentially be ensured through a PI3K-AKT manner.
- The activation of the A_1 adenosine receptor at **15 minutes post-reperfusion** is suggested to offer cardioprotection via the recruitment of the PI3K-AKT cell signalling pathway. This can be observed through the limiting infarct effects, a decrease in cell death (apoptosis and necrosis), and a decrease in cleaved caspase-3 activity and an increase in AKT phosphorylation through western blots. These cardioprotective effects have been seen to be reversed with the PI3K inhibitor Wortmannin.
- Activation of the A_1 adenosine receptor at **30 minutes post-reperfusion** is suggested to offer cardioprotection via the recruitment of the PI3K-AKT cell signalling pathway. This can be observed through the limiting infarct effects, a decrease in cell death (apoptosis and necrosis), and a decrease in cleaved caspase-3 activity and an increase in AKT phosphorylation through western blots. These cardioprotective effects have been seen to be reversed with PI3K inhibitor Wortmannin.

Chapter 6: Administration of A_1 adenosine receptor agonist, 2'-MeCCPA (10nM) to protect the myocardium from ischaemia-reperfusion injury via the recruitment of the MEK1/2-ERK1/2 cell signalling pathway at the onset of reperfusion compared to 15 minutes and 30 minutes post-reperfusion

6.1 Introduction

The ERK1/2 signalling cascade belong to the family of the mitogen-activated protein kinases (MAPKs); these are a family of serine-threonine kinases and can be implicated with the regulation of cell proliferation, differentiation and survival. They generally become activated in response to the occupation of the tyrosine kinase and G-protein-coupled receptors (Wildmann et al. 1999; Hausenloy and Yellon 2004). The ERK1/2 signalling cascade has the ability to be activated in an ischaemia-reperfusion setting and has been shown to mediate cellular protection by cardioprotective agents (Hausenloy and Yellon 2004).

There are different members of the MAP kinase family and these include the extracellular signal-regulated kinases (ERK), p38 MAP kinases and JNKs (Singh et al. 2018). Studies have also shown that MAP kinases and MEK can play a major role within cardioprotection within the phenomenon of ischaemic pre-conditioning (da Silva et al. 2004; Singh et al. 2018).

Previous literature has shown the activation of A_1, A_2 and A_3 adenosine receptor activation can be associated with the phosphorylation of the ERK1/2 kinase cascade within Chinese Hamster ovary cell model (Schulte and Fredholm 2000). Further research by Baxter et al. (2000), using the in vivo rabbit heart model of ischaemia-reperfusion injury, found that A_1/A_{2A} receptor agonist AMP579 had the ability to induce cardioprotection when administered throughout reperfusion, this protection was abrogated in the presence of MEK1/2 inhibitor PD098059 (Baxter et al. 2000). This previously reported literature can further suggest that upon the activation of the A_1 adenosine receptor, cardioprotection could be conferred via the recruitment of the MEK1/2-ERK1/2 cell signalling pathway. A study conducted by Zhao et al. (2001) demonstrated that preconditioning with A_1 adenosine receptor agonist CCPA, reduced infarct size and increase phosphorylation of p38-MAP kinase which therefore suggests cardioprotective effects. Germack and Dickenson (2005) also reported that preconditioning

neonatal rat cardiomyocytes with A_1 adenosine agonist CPA was also able to protect the myocardium from ischaemia-reperfusion injury via the recruitment of the MEK1/2-ERK1/2 signalling pathway. When MEK1 inhibitor (PD98059) was administered, this reversed all cardioprotective effects which confirmed that the A_1 adenosine receptor, upon activation, can ensure cardioprotection via the activation of the MEK1/2-ERK1/2 signalling pathway.

Further research conducted by Maddock et al. (2002) examined the role of the A_3 adenosine receptor in cardioprotection within the reperfusion phase in isolated rat cardiomyocyte models. It was found that the A_3 adenosine receptor agonist 2-Cl-IB-MECA, when administered throughout reperfusion was able to limit infarct size and significantly decrease apoptosis and necrosis. Furthermore, Hussain et al. (2014) assessed the cardioprotective effects of 2-CI-IB-MECA via the recruitment of the MEK1/2-ERK1/2 cell signalling pathway and it was found that this pro-survival signalling pathway played a vital role in decreasing caspase-3 levels and mediating cardioprotection. With such extensive research conducted into A_3 adenosine receptor mediated cardioprotection when activated throughout the reperfusion phase, this meant that more research needed to be conducted into the A_1 adenosine receptor being activated at reperfusion and throughout reperfusion rather than through the preconditioning phenomenon.

6.1.1 Aims and Objectives

1. To determine the cardioprotective effects of the A_1 adenosine receptor agonist, 2'-MeCCPA (10nM) in association with the recruitment of the pro-survival MEK1/2-ERK1/2 cell signalling pathway. A_1AR agonist 2'-MeCCPA (10nM) was administered at the onset of reperfusion in the presence and absence of MEK1/2 inhibitor UO126 (10µM) in order to assess infarct size (%), cell death (apoptosis and necrosis), p-ERK phosphorylation in Western blot and caspase-3 activity.
2. To determine the cardioprotective effects of the A_1 adenosine receptor agonist, 2'-MeCCPA (10nM) in association with the post-reperfusion/reoxygenation recruitment of the pro-survival MEK1/2-ERK1/2 cell signalling pathway. A_1AR agonist 2'-MeCCPA (10nM) was administered at 15 minutes post-reperfusion/reoxygenation in the

presence and absence of MEK1/2 inhibitor UO126 in order to assess infarct size (%), cell death (apoptosis and necrosis) and caspase-3 activity.

3. To determine the effects of A_1AR agonist 2'-MeCCPA (10nM) in the association of with the post-reperfusion/reoxygenation recruitment of the pro-survival MEK1/2-ERK1/2 cell signalling pathway. 2'-MeCCPA (10nM) was administered at 30 minutes post-reperfusion/reoxygenation in the presence and absence of MEK1/2 inhibitor UO126 in order to assess infarct size (%), cell death (apoptosis and necrosis) and caspase-3 activity.

6.2 Methods

6.2.1 Chemicals

2'-MeCCPA and UO126 were both supplied from Tocris Cookson (Bristol) and prepared in the same manner as described in Chapter 2, Section 2.2.

6.2.2 Animals

Adult male Sprague-Dawley rats (350 ± 50g) were supplied from Charles River (UK). Animals all received human care and assistance and were sacrificed by cervical dislocation as outlined in the Schedule 1 Home Office Procedure in accordance with the Scientific Procedure Act 1986. This process is described in Chapter 2, Section 2.1.

6.2.3 Langendorff protocol – Isolated perfused rat heart preparation

Briefly, all experiments using the Langendorff technique were carried out for 175 minutes (full details in Chapter 2, Section 2.3.5). Hearts were given 20 minutes for stabilisation, 35 minutes for simulated regional ischaemia followed by 120 minutes of reperfusion. Hearts were then randomly allocated into the following treatment groups:

a) Normoxic control – Rat hearts were perfused with KH buffer for 175 minutes (no simulated ischaemia induced here).

b) Ischaemia-reperfusion (IR) control – Isolated rat hearts were perfused with KH buffer for 20 minutes followed by 35 minutes of simulated regional ischaemia and 120 minutes of reperfusion.
c) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of MEK1/2 inhibitor UO126 (10µM) at the onset of reperfusion – Rats were perfused with KH buffer for 20 minutes, 35 minutes of simulated regional ischaemia followed by 120 minutes of reperfusion where drug administration occurred at the onset of reperfusion.
d) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of MEK1/2 inhibitor UO126 (10µM) at 15 minutes post-reperfusion - Rats were perfused with KH buffer for 20 minutes, 35 minutes of simulated regional ischaemia followed by 120 minutes of reperfusion where drug administration occurred at 15 minutes post-reperfusion.
e) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of MEK1/2 inhibitor UO126 (10µM) at 30 minutes post-reperfusion - Rats were perfused with KH buffer for 20 minutes, 35 minutes of simulated regional ischaemia followed by 120 minutes of reperfusion where drug administration occurred at 30 minutes post-reperfusion.

Throughout the Langendorff experiments, the stability of the hearts were consistently monitored via haemodynamic parameters of the heart rate (HR), left ventricular developed pressure (LVDP) and coronary flow (CF) (as described in Chapter 2, Section 2.3.2).

Following the reperfusion period, the infarct size to risk ratio was assessed using Evans blue triphenyltetrazolium chloride (TTC) staining procedures as described in Chapter 2, Section 2.3.6.

6.2.4 Isolation of adult rat ventricular cardiomyocytes

Isolation of adult rat ventricular cardiomyocytes was previously described in Chapter 2, Section 2.4.

6.2.5 Induction of hypoxia and reoxygenation conditions in adult rat cardiomyocytes

Fully described in Chapter 2, Section 2.4.1.

6.2.6 Experimental drug treatment protocol in adult rat ventricular cardiomyocytes

Isolated rat cardiomyocytes were exposed to different control and drug treatments. All experimental conditions for this chapter are as detailed below:

a) Normoxic control – Isolated myocytes were exposed to normoxic conditions for a total of 4 hours at 37^{o}C, 5% CO_2 and 95% O_2.
b) Hypoxia-Reoxygenation control – Isolated cardiomyocytes were exposed to 1 hour of hypoxia conditions followed by the onset of reoxygenation for 3 hours.
c) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of MEK1/2 inhibitor UO126 (10µM) at the onset of reoxygenation – Isolated cardiomyocytes were exposed to 1 hour hypoxia conditions followed by drug treatment at the onset of reoxygenation for 3 hours at 37^{o}C, 5% CO_2 and 95% O_2.
d) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of MEK1/2 inhibitor UO126 (10µM) at 15 minutes post-reoxygenation – Isolated cardiomyocytes were exposed to 1 hour hypoxia conditions followed by drug treatment at 15 minutes post-reoxygenation for 3 hours at 37^{o}C, 5% CO_2 and 95% O_2.
e) A_1AR agonist 2'-MeCCPA (10nM) administered in the presence and absence of MEK1/2 inhibitor UO126 (10µM) at 30 minutes post-reoxygenation – Isolated cardiomyocytes were exposed to 1 hour hypoxia conditions followed by drug treatment at 30 minutes post-reoxygenation for 3 hours at 37^{o}C, 5% CO_2 and 95% O_2.

6.2.7 Quantitative Analysis of Cell Death using Dead Cell Apoptosis Kit with Annexin V FITC with Propidium Iodide (PI) in Adult Rat Cardiomyocytes

Followed by the drug treatments described above, isolated rat ventricular cardiomyocytes were assess for their levels of apoptosis and necrosis using the Dead Cell Apoptosis Kit with Annexin V FITC and PI was purchased from ThermoFisher (UK) (previously detailed in Chapter 2, Section 2.5.2). Data was normalised against the cell only control and the values obtained were calculated as a relative change in apoptosis and necrosis activity of the mean absorbance of the control group.

6.2.8 Analysis of Cleaved Caspase-3 Activity in Adult Rat Cardiomyocytes

Following the drug treatment protocol as described above, the rat cardiomyocytes were probed with cleaved caspase-3 antibody as described in Chapter 2, Section 2.5.1. Data obtained was normalised to the cell only control by subtracting the mean fluorescence background recorded in untreated samples. Data was presented as a relative change in fluorescence activity.

6.2.9 Data Analysis

All data that was presented in this project is expressed at the mean ± standard error of the mean (SEM). IBM Statistical Package for Social Sciences (SPSS®) software was used to statistically analyse the data. The statistical tests currently used to analyse infarct sizes, band densities and cell population data was by one-way ANOVA accompanied by Fishers Protected Least Significant Difference (LSD) test for multiple comparisons. To assess the difference in the data sets, a p-value of $p<0.05$ was used to consider statistical significance.

Microsoft Excel was also used to present all data graphically.

6.3 Results

6.3.1 Profiling the effects of the administration of 2'-MeCCPA (10nM) at the onset of reperfusion/reoxygenation in the presence and absence of MEK1/2-ERK1/2 inhibitor, UO126 (10µM) and its effects upon isolated rat myocardium model and isolated rat cardiomyocytes

6.3.1.1 Haemodynamic Data – Profiling the effects of 2'-MeCCPA in the presence and absence of MEK1/2-ERK1/2 inhibitor, U0126 (10µM) on haemodynamic parameters (left ventricular developed pressure, heart rate and coronary flow).

Within this section, all hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and a further 120 minutes of reperfusion where 2'-MeCCPA (10nM) was administered alone firstly and then further administered in conjunction with U0126 (10µM) (MEK1/2-ERK1/2 inhibitor). U0126 (10µM) was also administered alone also. All treatments were administered at the onset of reperfusion to profile the effects of this inhibitor upon the A_1 adenosine receptor agonist, 2'-MeCCPA (10nM).

Overall, it was found that there was no significant difference between the groups at any of the time-points within the reperfusion period ($p>0.05$) (Figure 6.1).

Throughout the ischaemic period, all treatment groups had a significantly decreased LVDP in comparison to the normoxic control ($p<0.05$) (Figure 6.1).

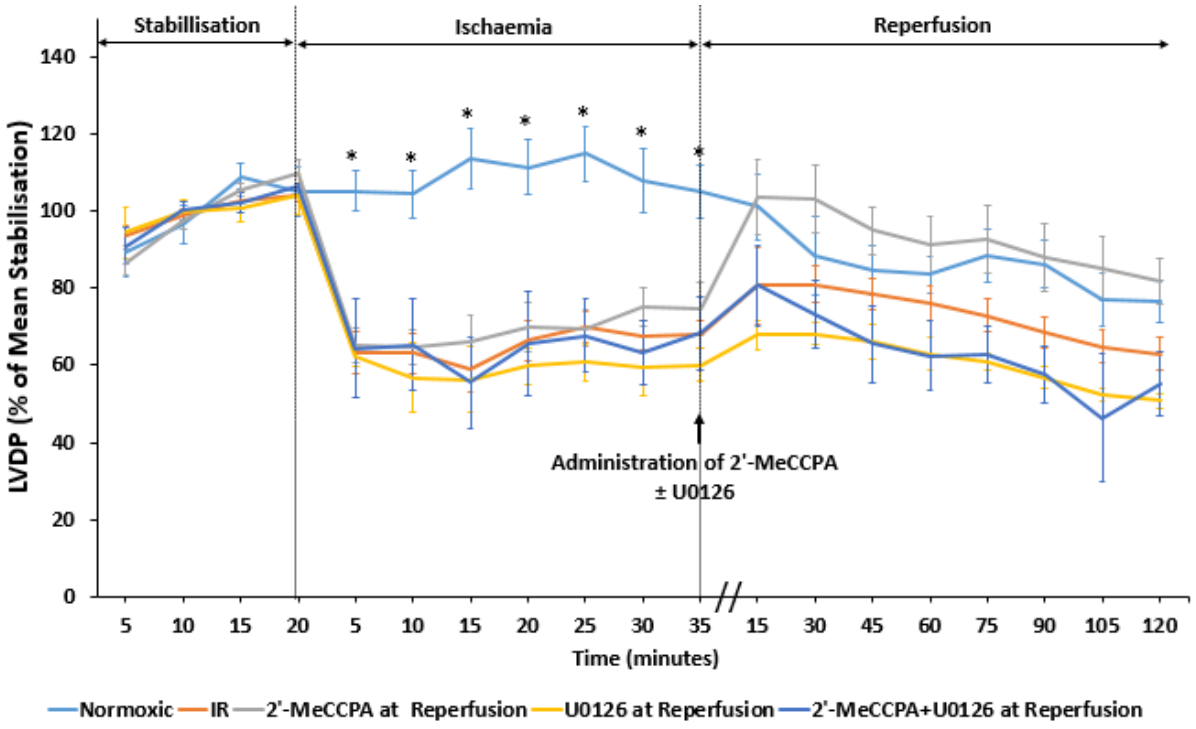


Figure 6. 1 Effects of 2'-MeCCPA (10nM) in the presence and absence of U0126 (10µM) on the left ventricular developed pressure within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at the onset of reperfusion in the presence and absence of U0126 (10µM). Data was presented as Mean±SEM, n=6-8. * $p<0.05$ All groups vs. Normoxia (ischaemia).

There was overall no significant difference detected on heart rate between treatment groups at all time-matched points ($p<0.05$) (Figure 6.2).

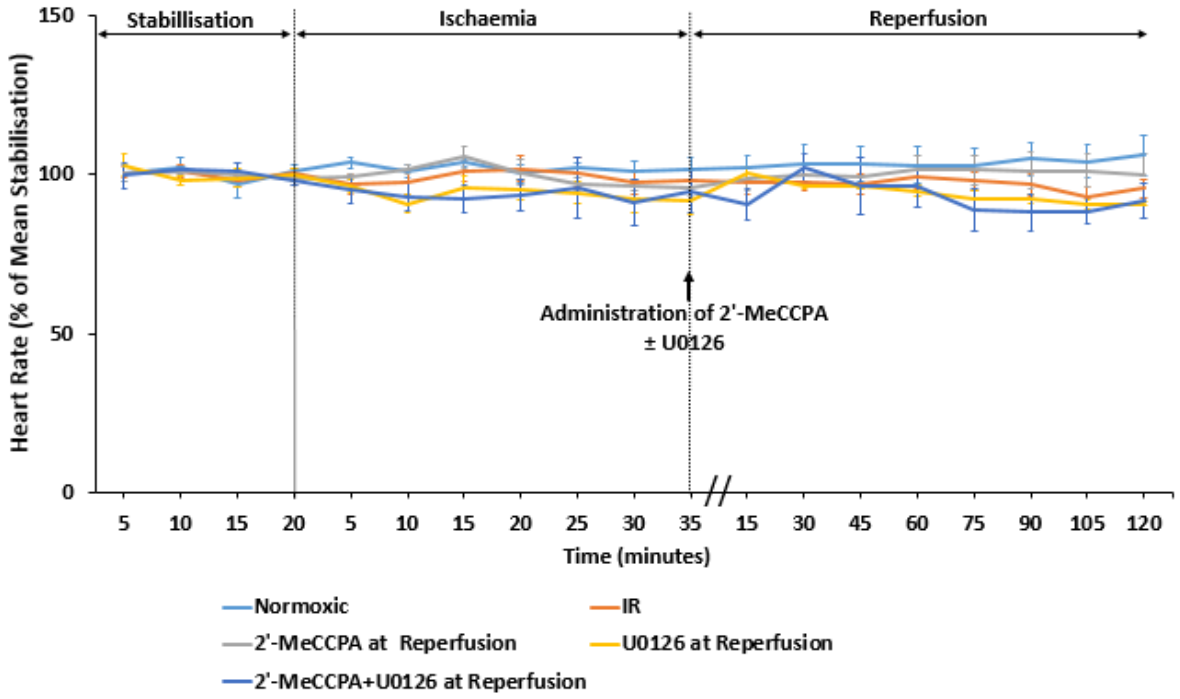


Figure 6. 2 Effects of 2'-MeCCPA (10nM) in the presence and absence of U0126 (10µM) on the heart rate within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at the onset of reperfusion in the presence and absence of U0126 (10µM). Data was presented as Mean±SEM, n=6-8.

No significant difference was detected between treatment groups at all time matched points within the reperfusion period ($p<0.05$) (Figure 6.3), however throughout the ischaemic period, all treatment groups had a significantly decreased coronary flow in comparison to the normoxic control ($p<0.05$) (Figure 6.3).

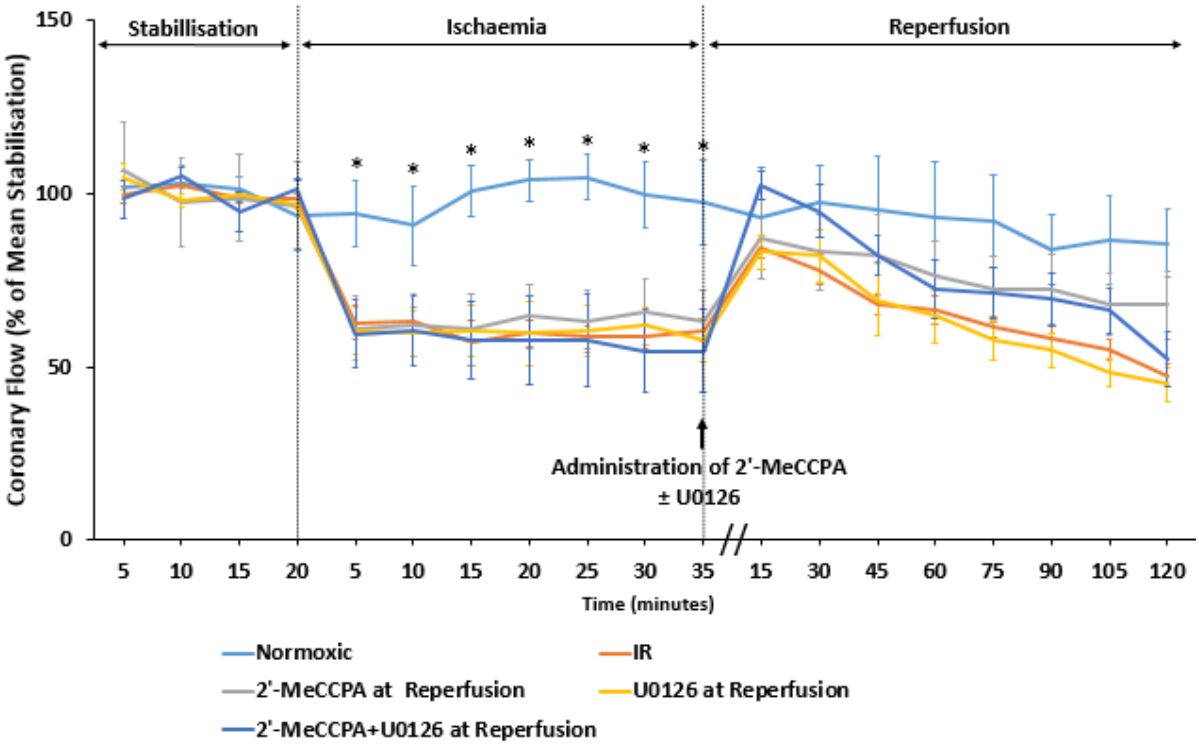


Figure 6. 3 Effects of 2'-MeCCPA (10nM) in the presence and absence of U0126 (10µM) on the coronary flow within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered at the onset of reperfusion in the presence and absence of U0126 (10µM). Data was presented as Mean±SEM, n=6-8. * $p<0.05$ All groups vs. Normoxia (ischaemia).

6.3.1.2 Profiling the effects of 2'-MeCCPA (10nM) with MEK1/2-ERK1/2 signalling pathway inhibitor, U0126 (10µM) on infarct size to risk ratio within isolated hearts subjected to ischaemia reperfusion injury at the onset of reperfusion.

All hearts were subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. A_1 adenosine receptor agonist, 2'-MeCCPA (10nM), was firstly administered alone and then co-administered in conjunction with MEK1/2-ERK1/2 signalling

pathway inhibitor, U0126 (10µM). U0126 was then further administered alone also. All treatments were administered at the onset of reperfusion to profile their effects upon infarct size to risk ratio (%).

A significant decrease in infarct size to risk ratio was detected when 2'-MeCCPA (10nM) was administered alone to the heart when compared with the IR control (28 ± 4% vs. 55 ± 6%, $p<0.001$). A significant decrease in infarct size to risk ratio (%) was also detected when 2'-MeCCPA (10nM) + U0126 (10µM) was administered together when compared to the IR control (35 ± 4% vs. 55 ± 6%, $p<0.01$). When 2'-MeCCPA + U0126 was administered together compared to 2'-MeCCPA being administered alone, a significant increase as shown here (retrospectively, 45 ± 2% vs. 28 ± 4%, $p<0.05$) (Figure 6.4).

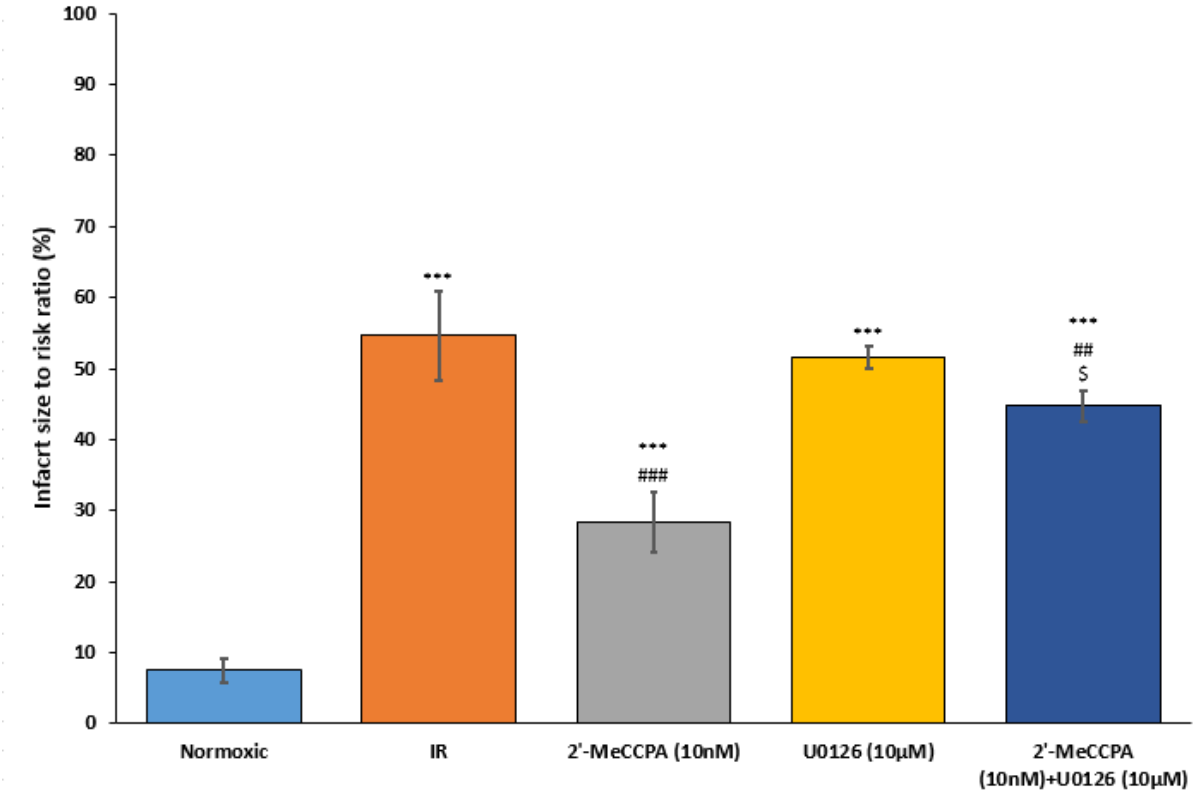


Figure 6. 4 Infarct size to risk ratio (%) within isolated perfused hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion subjected to 2'-MeCCPA (10nM) administered alone as well as when U0126 (10µM) (MAP1/2-ERK1/2 signalling pathway inhibitor) administered alone and 2'-MeCCPA + U0126 co-administered together at the onset of reperfusion. Data presented as Mean±SEM. n=6-8. *** $p<0.001$ vs. normoxic, ### $p<0.01$ vs. IR, ## $p<0.01$ vs IR and # $p<0.05$ vs. IR.

6.3.1.3 The role of MEK-ERK1/2 cell signalling pathway in 2'-MeCCPA mediated cardioprotection when administered at the onset of reperfusion within rat cardiomyocytes subjected to 1 hours of hypoxia and 3 hours of reoxygenation on apoptosis and necrosis

Administration of A_1 adenosine receptor agonist 2'-MeCCPA (10nM) at reoxygenation was significantly able to decrease the number of apoptotic and necrotic cardiomyocytes when compared to the non-treated Hyp/Reox group (Figure 6.5). In order to determine the mechanisms involved within 2'-MeCCPA (10nM) mediated cardioprotection, the role of the MEK-ERK1/2 cell signalling pathway was carried out using the MEK-ERK1/2 inhibitor U0126 (10µM). Isolated cardiomyocytes were subjected to 1 hour of hypoxia as well as a further 3 hours of reoxygenation. Throughout the reoxygenation period, cardiomyocytes were incubated with 2'-MeCCPA (10nM) in the presence and absence of MEK-ERK1/2 inhibitor U0126 (10µM).

The administration of A_1 adenosine receptor agonist 2'-MeCCPA (10nM) in the presence of MEK-ERK1/2 inhibitor U0126 (10µM) attenuated the anti-apoptotic effect of 2'-MeCCPA (10nM) in comparison to when 2'-MeCCPA (10nM) was administered alone throughout reoxygenation, this was not a significant change (23±3% 2'-MeCCPA + U0126 at onset of reoxygenation vs. 16±4% 2'-MeCCPA at the onset of reoxygenation, $p<0.05$) (Figure 6.5). The administration of 2'-MeCCPA (10nM) throughout reoxygenation in the presence of U0126 (10µM) did however significantly attenuate the anti-necrotic effects of 2'-MeCCPA (10nM) compared to when 2'-MeCCPA (10nM) was administered alone throughout reoxygenation (23±3% 2'-MeCCPA + U0126 onset of Reox vs. 13±2% 2'-MeCCPA onset of Reox, $p<0.001$) (Figure 6.5).

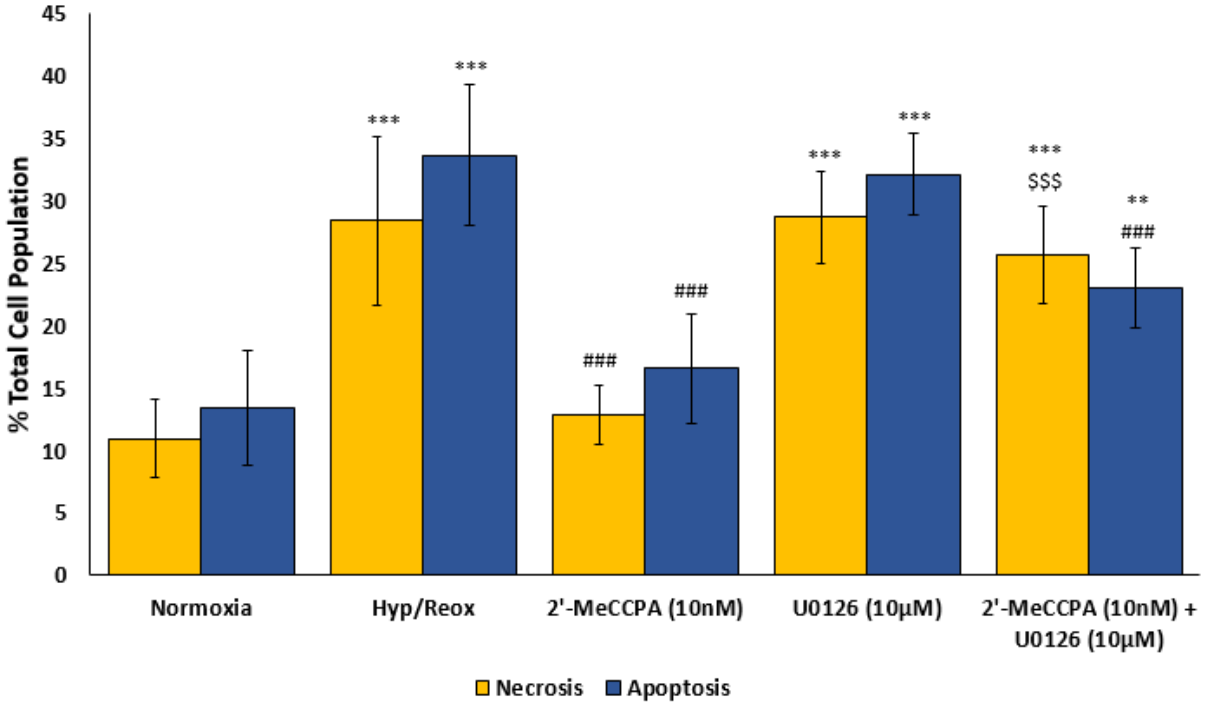


Figure 6. 5 Assessment of apoptosis and necrosis within isolated rat cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation. 2'-MeCCPA (10nM) was administered at the onset of reoxygenation in the presence and absence of MEK-ERK1/2 inhibitor, U0126 (10µM). Results were shown as Mean±SEM and were expressed as a percentage of 10 000 cells counted from 6 individual experiments. *** $p<0.001$ vs. Normoxia. ** $p<0.01$ vs. Normoxia. ### $p<0.001$ vs. Hyp/Reox. $$$ $p<0.001$ vs. 2'-MeCCPA (10nM).

6.3.1.4 The role of MEK/ERK1/2 cell signalling pathway on cleaved-caspase 3 activity upon administration of 2'-MeCCPA (10nM) at reoxygenation in isolated adult rat cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation

The administration of 2'-MeCCPA (10nM) at reoxygenation significantly decreased cleaved-caspase 3 activity when compared to cardiomyocytes undergoing 1 hour hypoxia and a further 3 hours of reoxygenation (Hyp/Reox) (retrospectively, 181±35% vs. 313±35%, $p<0.001$) (Figure 6.6). To further determine whether the decrease in cleaved-caspase 3 activity by 2'-MeCCPA (10nM) involved the MEK-ERK1/2 cell signalling pathway, the MEK-ERK1/2 inhibitor U0126 (10µM) was utilised. The isolated cardiomyocytes were subjected to 1 hour of hypoxia and 3 hours of reoxygenation where 2'-MeCCPA (10nM) was administered at reoxygenation in the presence and absence of MEK-ERK1/2 inhibitor U0126 (10µM).

The administration of A_1 adenosine receptor agonist 2'-MeCCPA (10nM) in the presence of MEK-ERK1/2 inhibitor U0126 (10µM) throughout reoxygenation significantly increased cleaved-caspase 3 activity compared to when 2'-MeCCPA (10nM) was administered alone at reoxygenation (retrospectively 285±39% vs. 181±35%, p>0.01) (Figure 6.6).

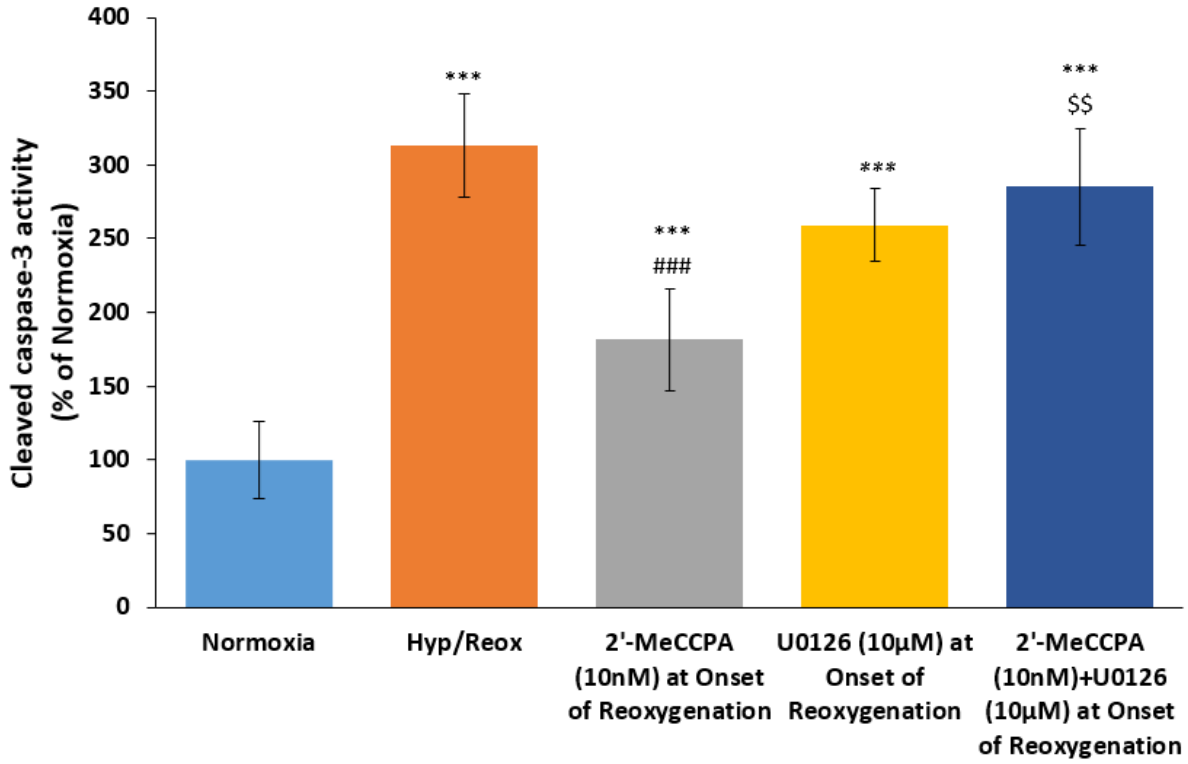


Figure 6. 6 Cleaved-caspase 3 activity within isolated rat cardiomyocytes subjected to 4 hours of reoxygenation (Normoxia) or 1 hour of hypoxia followed by 3 hours of reoxygenation (Hyp/Reox). 2'-MeCCPA (10nM) was administered at the onset of reoxygenation in the presence and absence of MEK-ERK1/2 cell signalling inhibitor U0126 (10µM). Mean±SEM on 6 individual experiments. *** p<0.001 vs. Normoxia. ### p<0.001 vs. Hyp/Reox, $$ p<0.01 vs. 2'-MeCCPA@Onset of Reox.

6.3.2 Effects of delayed administration of 2'-MeCCPA (10nM) at 15 minutes and 30 minutes post-reperfusion/reoxygenation in the presence and absence of MEK1/2-ERK1/2 inhibitor, UO126 (10µM) and its effects upon isolated rat myocardium model and isolated rat cardiomyocytes

6.3.2.1a Effects of postponing the administration of 2'-MeCCPA (10nM) to 15 minutes post-reperfusion in the presence and absence of MEK1/2-ERK1/2 inhibitor, UO126 (10µM) on haemodynamic parameters (left ventricular developed pressure (LVDP), heart rate and coronary flow).

Throughout Langendorff studies, the left ventricular developed pressure, heart rate and coronary flow was constantly monitored throughout all experiments. Perfused hearts were subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. A_1 adenosine receptor agonist, 2'-MeCCPA (10nM) was administered in the presence and absence of MEK1/2-ERK1/2 inhibitor UO126 (10µM) at 15 minutes post reperfusion.

Throughout the period of ischaemia, significant decrease in LVDP was detected in all treatment groups compared to the normoxia control group at time-matched points ($p<0.05$) (Figure 6.2); however throughout the period of reperfusion, an overall general decline in LVDP was detected after 15 minutes of reperfusion. These changes were not significant ($p>0.05$, Figure 6.7).

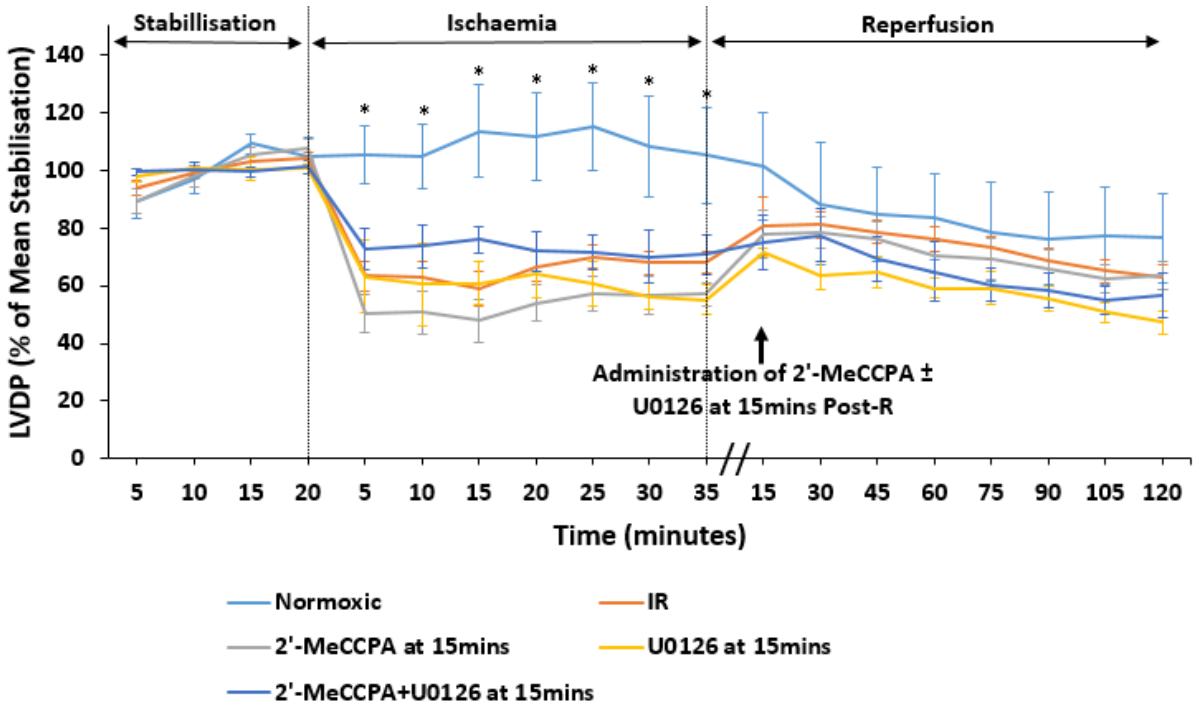


Figure 6. 7 Assessing the effects of 2'-MeCCPA (10nM) in the presence and absence of UO126 (10µM) when administered at 15 minutes post reperfusion on the left ventricular developed pressure (LVDP) in isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Data was presented as Mean±SEM, n=6-8. * $p<0.05$ All groups vs. Normoxia (ischaemia).

Overall, no significant effects were observed on heart rate between all time matched points within all treatment groups when 2'-MeCCPA (10nM) was administered in the presence or absence of UO126 (10µM) at 15 minutes post reperfusion ($p>0.05$, Figure 6.8).

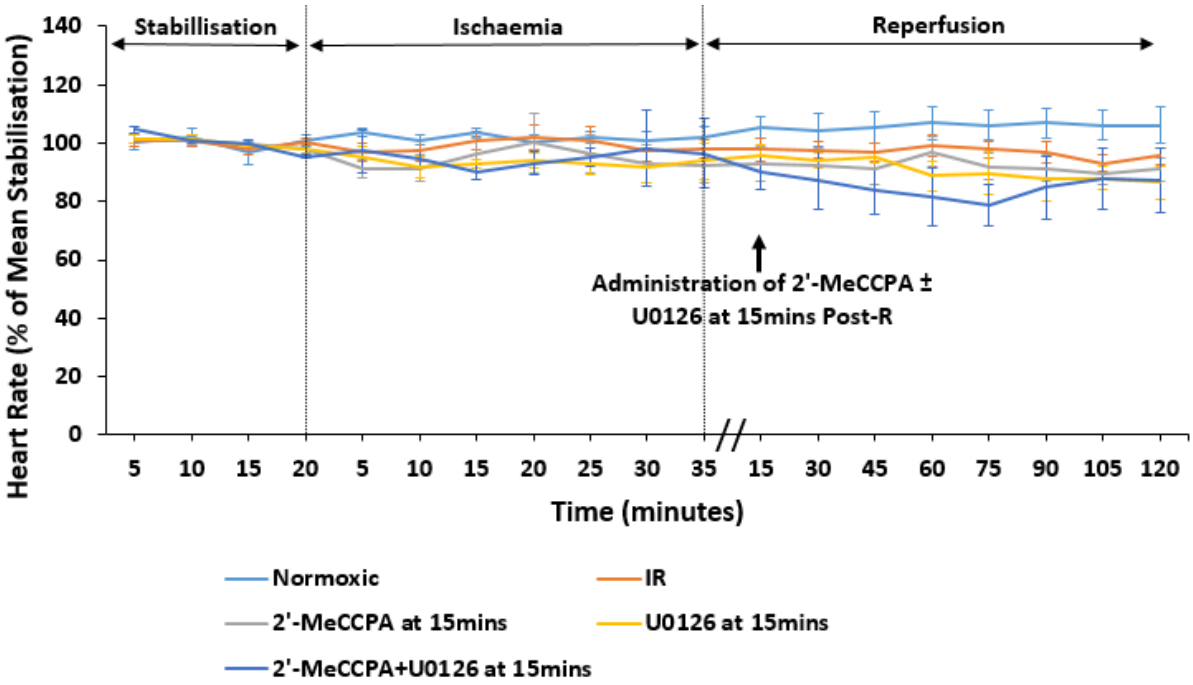


Figure 6. 8 Effects of the heart rate when 2'-MeCCPA (10nM), in the presence and absence of UO126 (10µM), when administered at 15 minutes post within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Data was presented as Mean±SEM, n=6-8.

Within the ischaemic period, the coronary flow for all treatment groups at each time matched points significantly decreased in comparison to the normoxic control ($p<0.05$, Figure 6.9). No significant changes were detected between treatments groups at all time matched points within the reperfusion period (Figure 6.9).

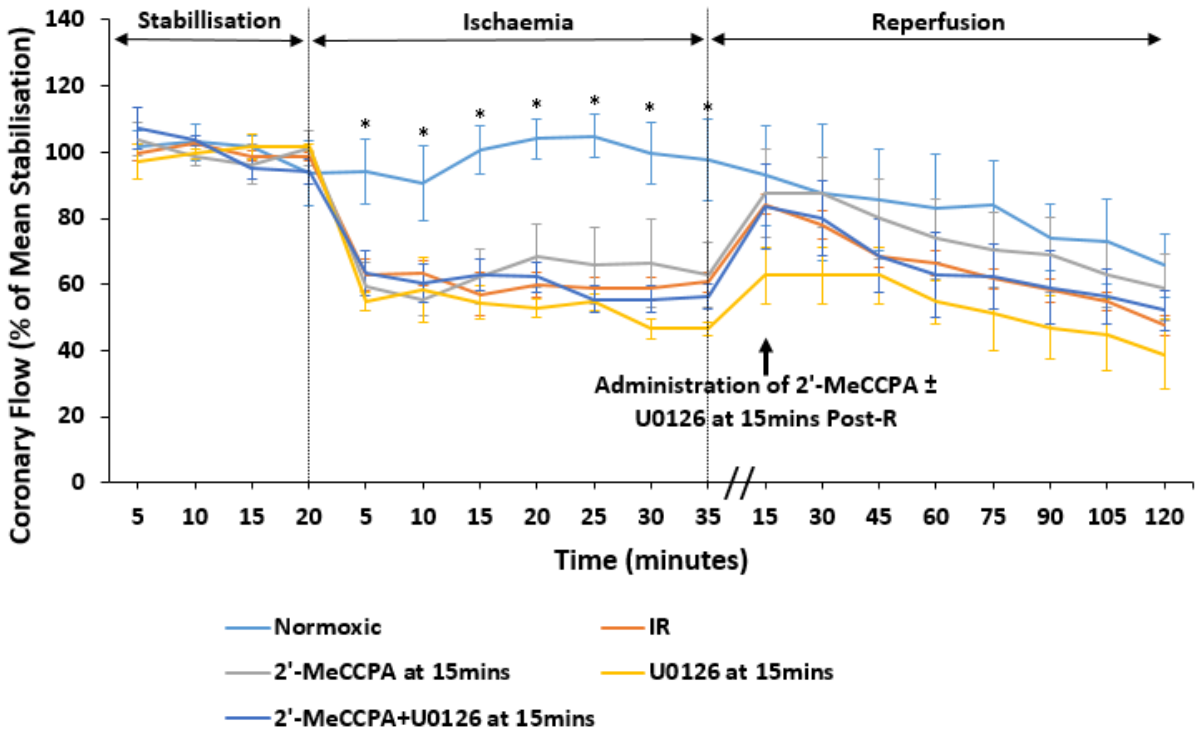


Figure 6. 9 Effects of coronary flow when 2'-MeCCPA (10nM, in the presence and absence of UO126 (10µM), when administered at 15 minutes post reperfusion within isolated rat hearts when subjected to 20 minutes stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Data was presented as Mean±SEM, n=6-8. * $p<0.05$ All groups vs. Normoxia (ischaemia).

6.3.2.1b Effects of postponing the administration of 2'-MeCCPA (10nM) to 30 minutes post-reperfusion in the presence and absence of MEK1/2-ERK1/2 inhibitor, UO126 (10µM) on haemodynamic parameters (left ventricular developed pressure (LVDP), heart rate and coronary flow).

It was previously shown that the administration of A_1 adenosine receptor agonist 2'-MeCCPA (10nM), when administered at the onset of reperfusion and 15 minutes post reperfusion can protect the ischaemic myocardium via the recruitment of the MEK1/2-ERK1/2 cell survival pathway. In order to further assess this cardioprotection, 2'-MeCCPA (10nM) was administered at 30 minutes post reperfusion in the presence and absence of MEK1/2 inhibitor UO126 (10µM) to determine if the MEK1/2-ERK1/2 cell survival pathway further enhanced cardioprotection at 30 minutes post reperfusion.

The left ventricular developed pressure, heart rate and coronary flow was constantly monitored throughout all Langendorff studies; and hearts were subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. 2'-MeCCPA (10nM) was administered in the presence and absence of UO126 (10µM) at 30 minutes post reperfusion.

It was observed that during the ischaemic period, all treatment groups at time matched points significantly decreased the LVDP in comparison to the normoxic heart group ($p<0.05$, Figure 6.10). Throughout the period of reperfusion, there was an overall decline in LVDP however there was no significant difference between treatment groups at time matched points ($p>0.05$, Figure 6.10).

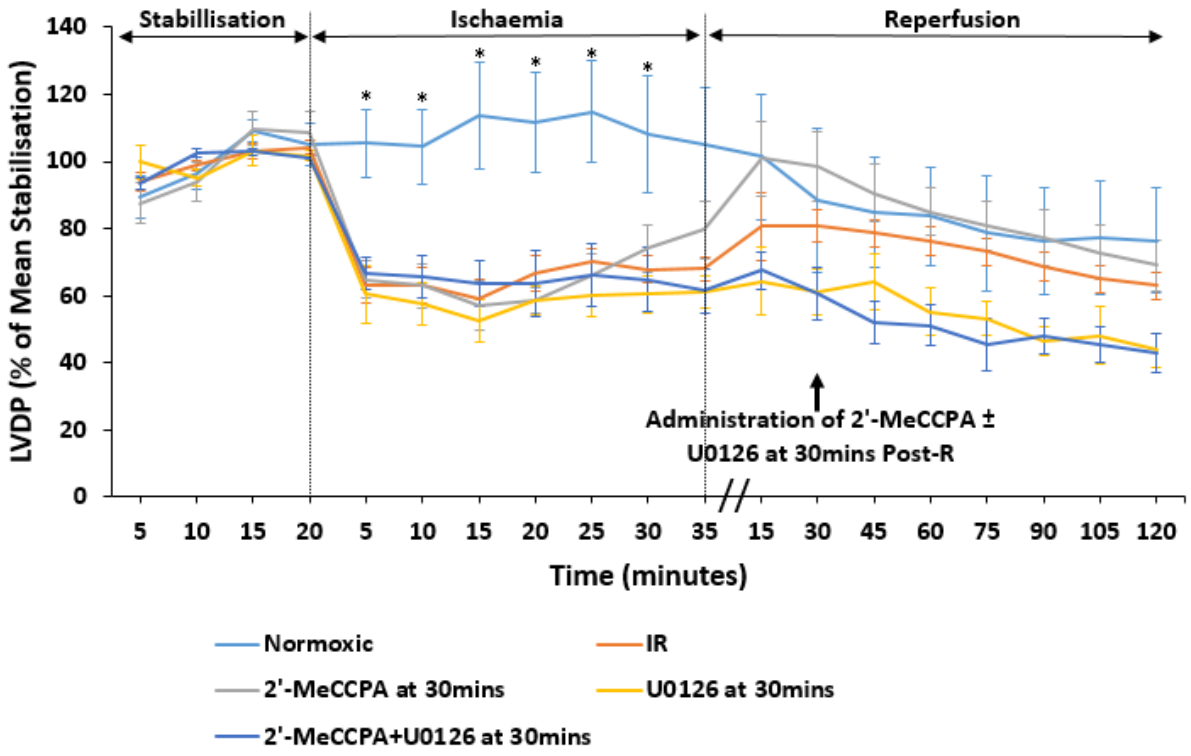


Figure 6. 10 Assessing the effects on left ventricular developed pressure when 2'-MeCCPA (10nM) was administered in the presence and absence of UO126 (10µM) at 30 minutes post reperfusion. Isolated rat hearts were subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and a further 120 minutes of reperfusion. Data was presented as Mean±SEM, n=6-8. * $p<0.05$ All groups vs. Normoxia (ischaemia).

No significant effects were detected on heart rate between all time matched points between different treatment groups ($p<0.05$, Figure 6.11).

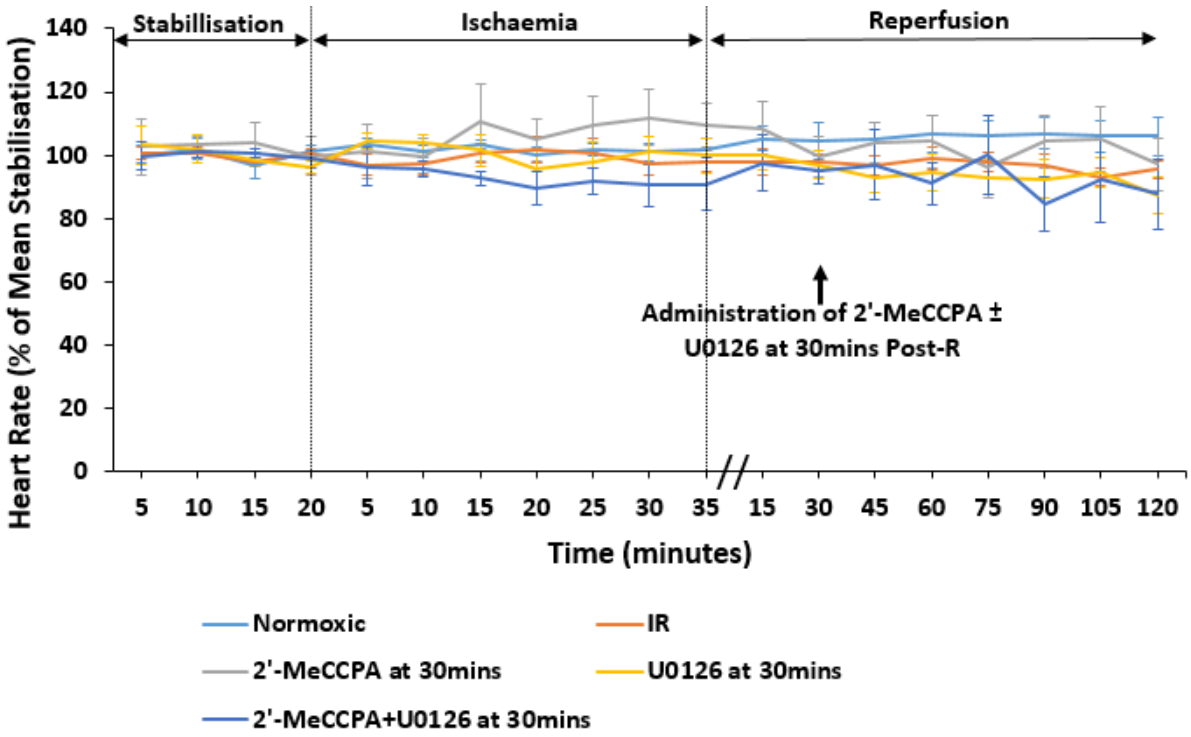


Figure 6. 11 The effects on heart rate when 2'-MeCCPA (10nM) was administered in the presence and absence of UO126 (10µM) at 30 minutes post reperfusion. Isolated rat hearts were subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and a further 120 minutes of reperfusion. Data was presented as Mean±SEM, n=6-8.

Overall, a significant decrease in coronary flow was detected in all treatment groups at all time matched points within the ischaemic period of the protocol in comparison to the normoxic control groups ($p<0.05$, Figure 6.12). No significant changes were observed throughout the reperfusion period ($p>0.05$, Figure 6.12).

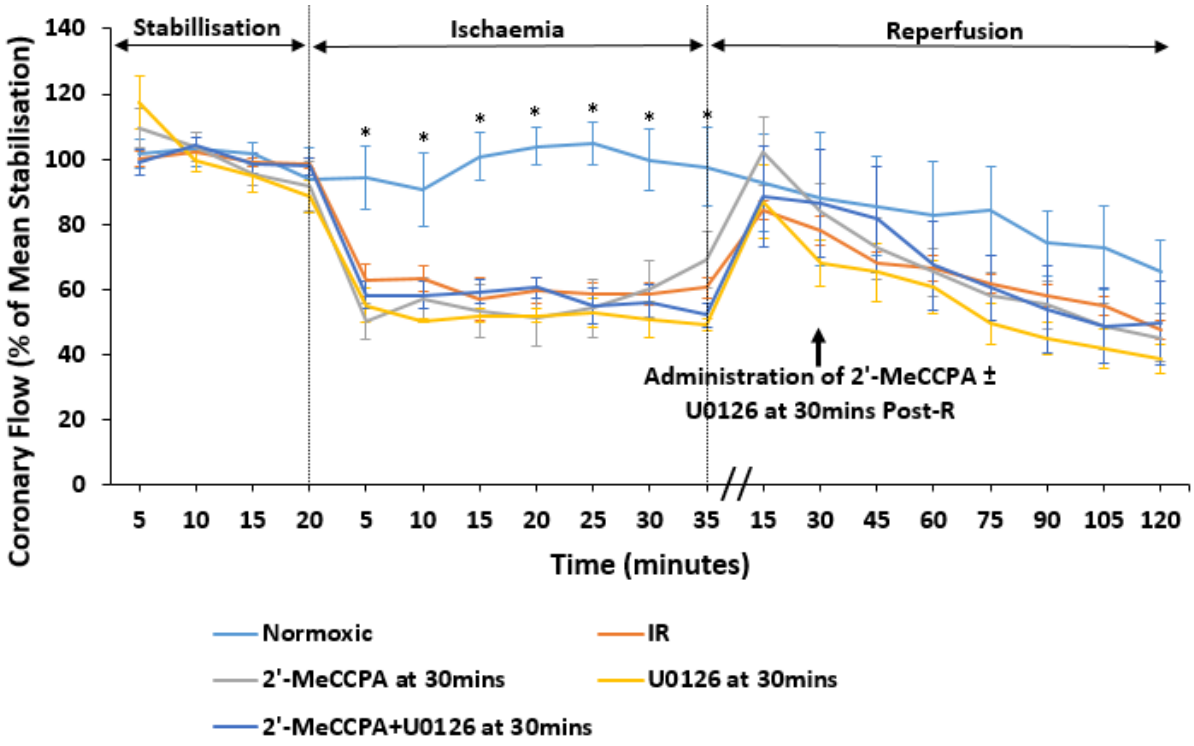


Figure 6. 12 Effects on coronary flow when 2'-MeCCPA (10nM) was administered in the presence and absence of UO126 (10µM) at 30 minutes post reperfusion. Isolated rat hearts were subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and a further 120 minutes of reperfusion. Data was presented as Mean±SEM, n=6-8. * $p<0.05$ All groups vs. Normoxia (ischaemia).

6.3.2.2a The effects of postponing the administration of 2'-MeCCPA (10nM) to 15 minutes post-reperfusion in the presence and absence of MEK1/2-ERK1/2 inhibitor, UO126 (10µM) on infarct size to risk ratio (%) within isolated hearts subjected to ischaemia reperfusion injury

It was previously shown that the administration of A_1 adenosine receptor agonist 2'-MeCCPA (10nM) at the onset of reperfusion was able to significantly protect the myocardium from ischaemia-reperfusion injury via the recruitment of the MEK1/2-ERK1/2 cell survival pathway. The involvement of the MEK1/2-ERK1/2 pathway was also assessed when 2'-MeCCPA (10nM) was administered at 15 minutes post reperfusion in the presence and absence of MEK1/2 inhibitor UO126 (10µM).

The administration of A_1 adenosine receptor agonist 2'-MeCCPA (10nM) at 15 minutes post reperfusion in the presence of MEK1/2 inhibitor UO126 (10µM) significantly abolished the cardioprotection detected when 2'-MeCCPA (10nM) was administered alone at 15 minutes post reperfusion (46±3% 2'-MeCCPA+UO126 at 15mins Post-R vs. 30±10% 2'-MeCCPA at 15mins Post-R, $p<0.05$) (Figure 6.13).

When the MEK1/2 inhibitor UO126 (10µM) was administered alone, there was no significant effect detected upon infarct size to risk ratio (%) when compared to the IR control (48±4% UO126 at 15mins Post-R vs. 55±6% IR control, $p>0.05$) (Figure 6.13).

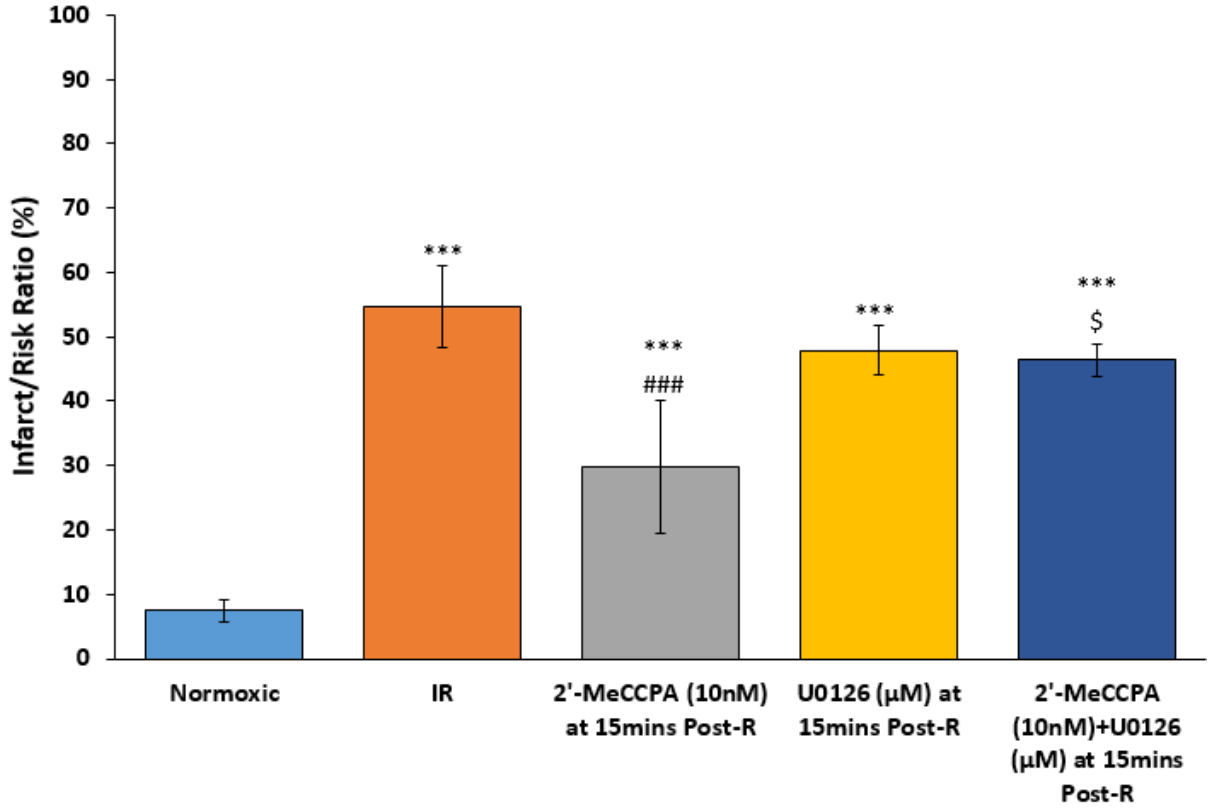


Figure 6. 13 The infarct size to risk ratio (%) within non-treated normoxic control, IR control and 2'-MeCCPA (10nM) treated ischaemic-reperfused hearts. All isolated perfused rat hearts were subjected to 20 minutes to stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. A_1 adenosine receptor agonist, 2'-MeCCPA (10nM) was administered in the presence and absence of MEK1/2 inhibitor, UO126 (10µM) at 15 minutes into the onset of reperfusion. *** $p<0.001$ vs. Normoxia. ### $p<0.001$ vs. IR control. $ $p<0.05$ vs. 2'-MeCCPA at 15mins Post-R.

6.3.2.2b The effects of postponing the administration of 2'-MeCCPA (10nM) to 30 minutes post-reperfusion in the presence and absence of MEK1/2-ERK1/2 inhibitor, UO126 (10µM) on infarct size to risk ratio (%) within isolated hearts subjected to ischaemia reperfusion injury.

The administration of A_1 adenosine receptor agonist 2'-MeCCPA (10nM) at 30 minutes post reperfusion, in the presence of MEK1/2 inhibitor UO126 (10µM) significantly abolished the 2'-MeCCPA (10nM) mediated cardioprotection as shown in Figure 6.14 (48±5% 2'-MeCCPA+UO126 at 30mins Post-R vs. 35±6% 2'-MeCCPA at 30mins Post-R, $p<0.05$) (Figure 6.15). When MEK1/2 inhibitor UO126 (10µM) was administered alone at 30 minutes post

reperfusion, there was no significant effect on infarct size to risk ratio (%) in comparison to the IR control (50±6% UO126 at 30mins Post-R vs. 55±6% IR control, p<0.05) (Figure 6.14).

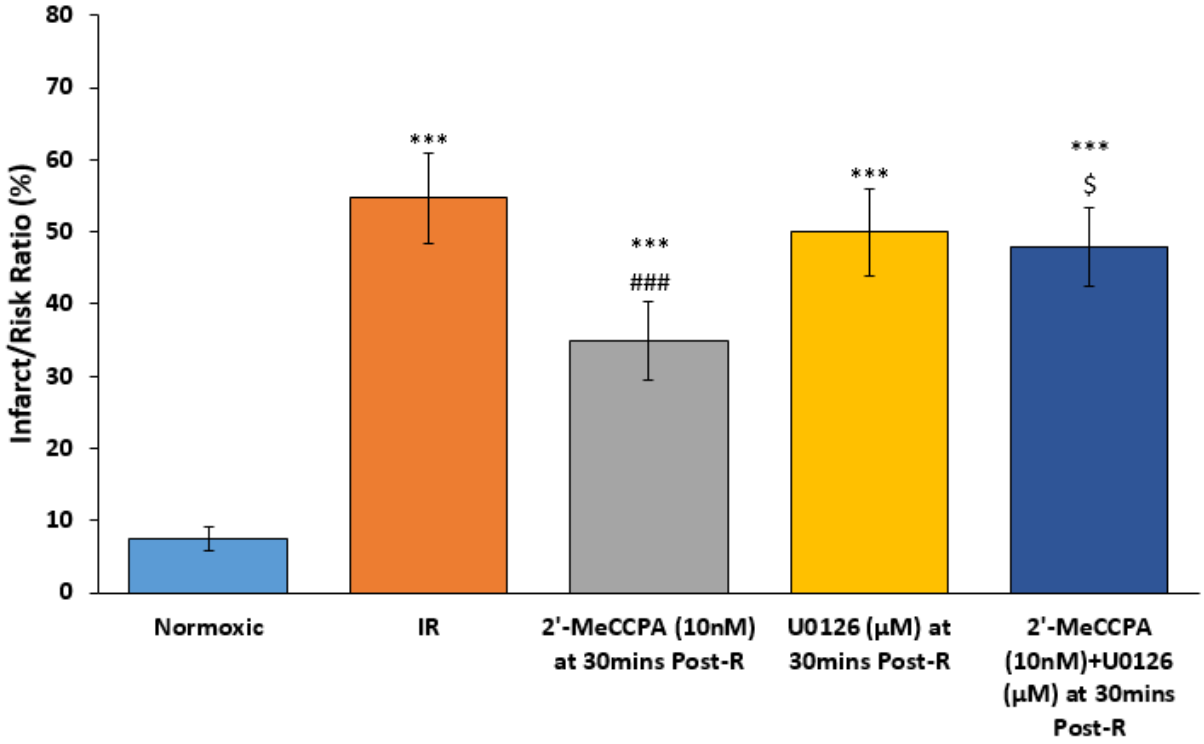


Figure 6. 14 The infarct size to risk ratio (%) within non-treated normoxic control, IR control and 2'-MeCCPA (10nM) treated ischaemic-reperfused hearts. All isolated perfused rat hearts were subjected to 20 minutes to stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. A_1 adenosine receptor agonist, 2'-MeCCPA (10nM) was administered in the presence and absence of MEK1/2 inhibitor, UO126 (10μM) at 30 minutes into the onset of reperfusion. *** p<0.001 vs. Normoxia. ### p<0.001 vs. IR control. $ p<0.05 vs. 2'-MeCCPA at 30mins Post-R.

6.3.2.3a Effects of postponing the administration of 2'-MeCCPA to 15 minutes post reoxygenation on isolated rat cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation from reoxygenation injury via the recruitment of the MEK1/2-ERK1/2 cell signalling pathway and its effects on cellular apoptosis and necrosis.

In previous chapters, it has been studied that when A_1 adenosine receptor agonist 2'-MeCCPA (10nM) is administered at 15 minutes into the onset of reoxygenation, a significant increase in cardioprotection of isolated perfused rat hearts was shown. This section studies whether the protection shown when 2'-MeCCPA (10nM) is administered at 15 minutes post

reoxygenation to cardiomyocytes that have undergone 1 hour of hypoxia and 3 hours of reoxygenation, is activated via the MEK1/2-ERK1/2 cell survival pathway.

Postponing the administration of the A_1 adenosine receptor agonist 2'-MeCCPA (10nM) to 15 minutes after reoxygenation significantly decreased the number of apoptotic cells (14±5% 2'-MeCCPA at 15mins Post-R vs. 34±6% Hyp/Reox, $p<0.001$) (Figure 6.15). Postponing the administration of 2'-MeCCPA (10nM) to 15 minutes post reoxygenation also significantly decreased the number of necrotic cells (16±4% 2'-MeCCPA at 15mins Post-R vs. 28±7% Hyp/Reox, $p<0.001$) (Figure 6.16).

When 2'-MeCCPA (10nM) was administered at 15 minutes post reoxygenation in the presence of MEK1/2 inhibitor UO126 (10µM), anti-apoptotic effects that were studied when 2'-MeCCPA (10nM) was administered alone at 15 minutes post reoxygenation were abolished (30±5% 2'-MeCCPA+UO126 at 15mins Post-R vs. 14±5% 2'-MeCCPA at 15mins Post-R, $p<0.001$) (Figure 6.15). The anti-necrotic effects observed when 2'-MeCCPA (10nM) was administered alone at 15 minutes post reoxygenation were significantly abolished when 2'-MeCCPA (10nM) as administered alongside MEK1/2 inhibitor UO126 (10µM) (23±3% 2'-MeCCPA+UO126 at 15mins Post-R vs. 16±4% 2'-MeCCPA at 15mins Post-R, $p<0.05$) (Figure 6.16).

When UO126 (10µM) was administered alone 15 minutes post reoxygenation, there was no significant effect observed upon apoptosis within cardiomyocytes compared to the Hyp/Reox group (30±4% UO126 at 15mins Post-R vs. 34±6% Hyp/Reox, $p>0.05$). (Figure 6.15). When UO126 (10µM) was administered alone at 15 minutes post reoxygenation, there was no significant effect observed upon necrosis within the cardiomyocytes in comparison to the Hyp/Reox group (24±3% UO126 at 15mins Post-R vs. 28±7% Hyp/Reox, $p>0.05$) (Figure 6.16).

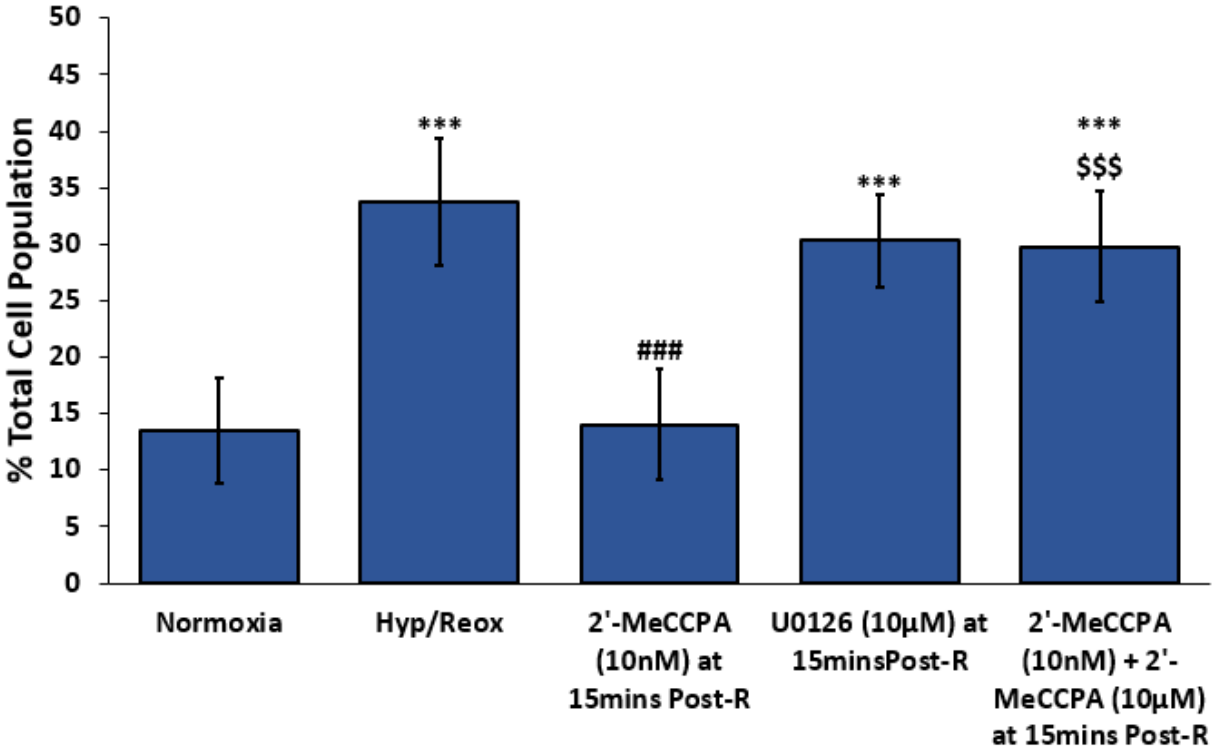


Figure 6. 15 The assessment of apoptosis in isolated adult rat cardiac myocytes that were subjected to 4 hours of reoxygenation (Normoxia) or 1 hour of hypoxia and 3 hours of reoxygenation (Hyp/Reox). The role of the MEK1/2-ERK1/2 cell survival pathway in ensuing 2'-MeCCPA mediated cardioprotection was assessed. The A_1AR agonist 2'-MeCCPA (10nM) was administered at 15 minutes post reoxygenation in the presence and absence of MEK 1/2 inhibitor UO126 (10µM). Results were shown as Mean±SEM and expressed as percentages of 10 000 total cells counted. *** p<0.001 vs. Normoxia. ### p<0.001 vs. Hyp/Reox. $$$ p<0.001 vs. 2'-MeCCPA at 15mins Post-R.

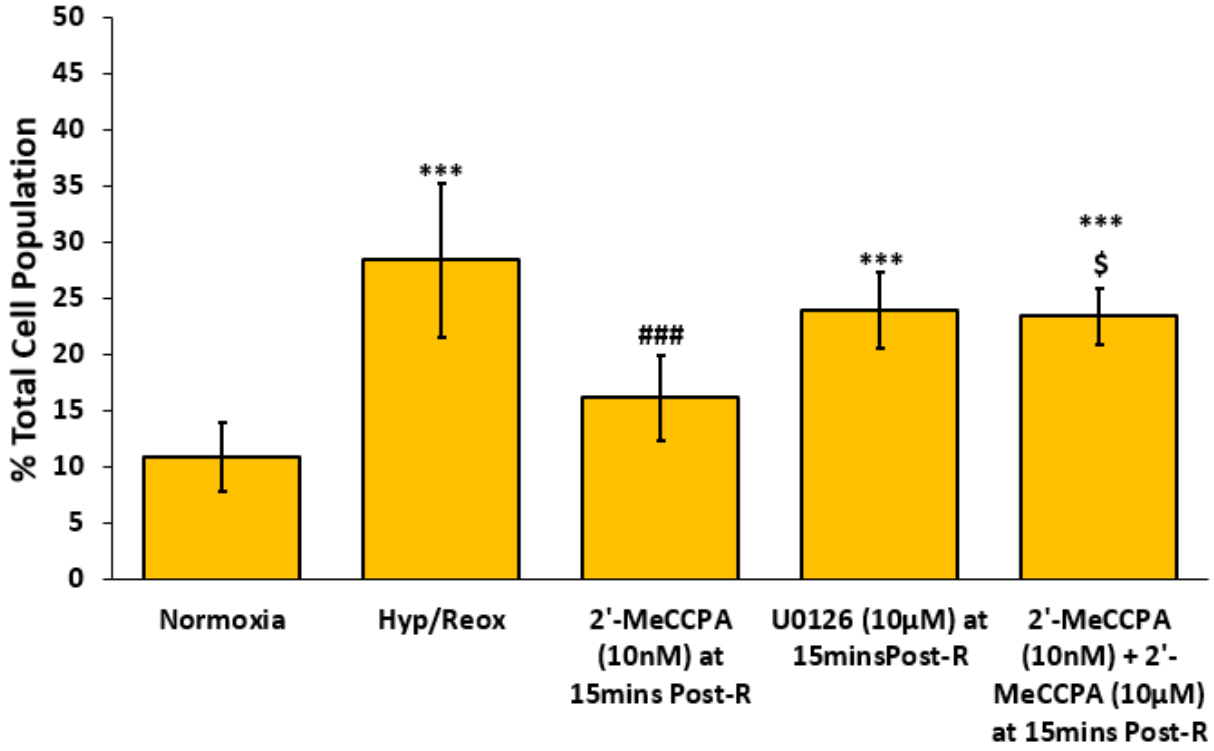


Figure 6. 16 The assessment of necrosis in isolated adult rat cardiac myocytes that were subjected to 4 hours of reoxygenation (Normoxia) or 1 hour of hypoxia and 3 hours of reoxygenation (Hyp/Reox). The role of the MEK1/2-ERK1/2 cell survival pathway in ensuing 2'-MeCCPA mediated cardioprotection was assessed. The A_1AR agonist 2'-MeCCPA (10nM) was administered at 15 minutes post reoxygenation in the presence and absence of MEK 1/2 inhibitor UO126 (10µM). Results were shown as Mean±SEM and expressed as percentages of 10 000 total cells counted. *** $p<0.001$ vs. Normoxia. ### $p<0.001$ vs. Hyp/Reox. $ $p<0.05$ vs. 2'-MeCCPA at 15mins Post-R.

6.3.2.3b Effects of postponing the administration of 2'-MeCCPA to 30 minutes post reoxygenation on isolated rat cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation from reoxygenation injury via the recruitment of the MEK1/2-ERK1/2 cell signalling pathway and its effects on cellular necrosis and apoptosis.

It was observed that when the administration of A_1 adenosine receptor agonist 2'-MeCCPA (10nM) was postponed to 30 minutes post reoxygenation, there was a significant decrease in cellular apoptosis when compared to the Hyp/Reox group (15±5% 2'-MeCCPA 30mins Post-R vs. 34±6% Hyp/Reox, $p<0.001$) (Figure 6.17). The postponement of the administration of A_1 adenosine receptor agonist 2'-MeCCPA (10nM) to 30 minutes post reoxygenation also

significantly decreased cellular necrosis when compared to the Hyp/Reox group (17±3% 2'-MeCCPA 30mins Post-R vs. 28±7% Hyp/Reox, $p<0.001$) (Figure 6.18).

The anti-apoptotic effect of the administration of 2'-MeCCPA alone at 30 minutes post reoxygenation were then significantly abolished when 2-MeCCPA was administered in the presence of MEK1/2 inhibitor UO126 (10µM) at 30 minutes post reoxygenation (35±3% 2'-MeCCPA+UO126 30mins Post-R vs. 15±5% 2'-MeCCPA 30mins Post-R, $p<0.001$) (Figure 6.17). It was also found that when 2'-MeCCPA (10nM) and UO126 (10µM) were administered in conjunction at 30 minutes post reoxygenation, there as a significant increase in cellular necrosis compared to when 2'-MeCCPA (10nM) was administered alone at 30 minutes post reoxygenation, the anti-necrotic effects were reversed when UO126 (10µM) were administered in conjunction (25±4% 2'-MeCCPA+UO126 30mins Post-R vs. 17±3% 2'-MeCCPA 30mins Post-R, $p<0.05$) (Figure 6.18). This was not a vast difference in necrosis however it was a significant difference.

When MEK1/2 inhibitor UO126 (10µM) was administered alone at 30 minutes reoxygenation, there was no significant effects observed on cellular apoptosis when compared to the Hyp/Reox group (30±4% UO126 30mins Post-R vs. 34±6% Hyp/Reox, $p>0.05$) (Figure 6.17). When UO126 (10µM) was administered alone at 30 minutes post reoxygenation, there was no significant effect observed compared to the Hyp/Reox control group (28±4% UO126 30mins Post-R vs. 28±7% Hyp/Reox, $p>0.05$) (Figure 6.18).

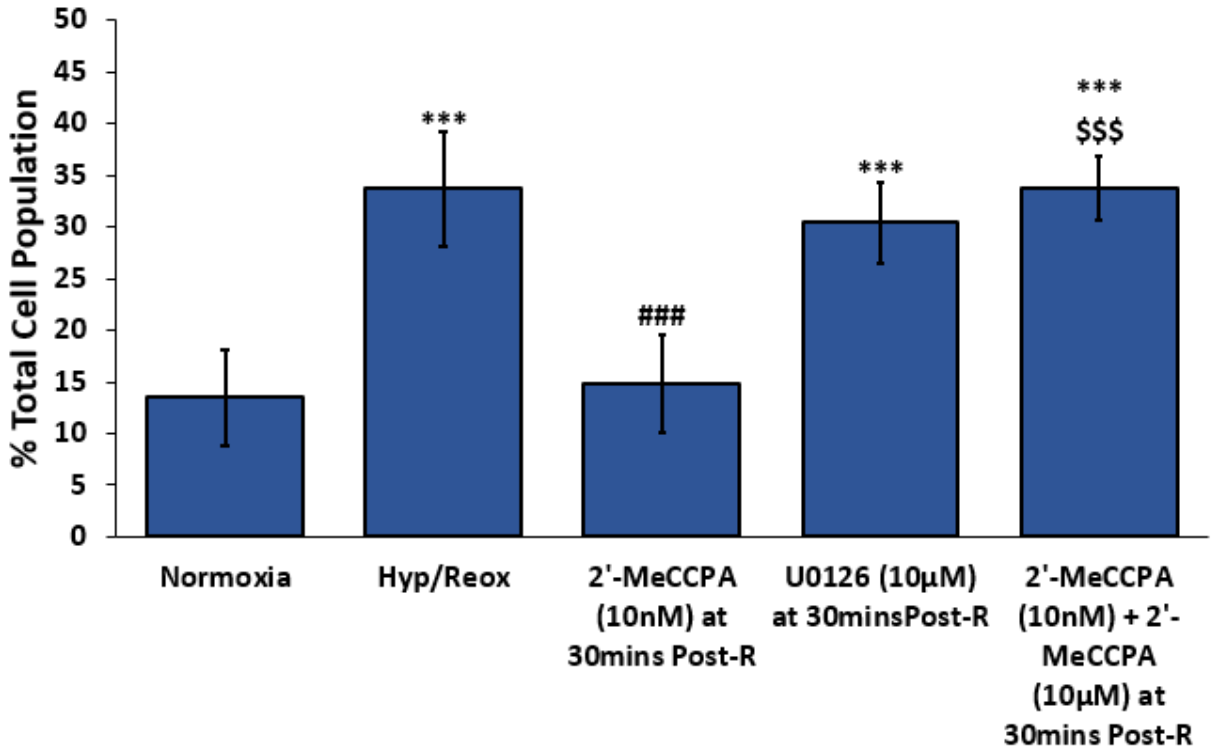


Figure 6. 17 The assessment of apoptosis in isolated adult rat cardiac myocytes that were subjected to 4 hours of reoxygenation (Normoxia) or 1 hour of hypoxia and 3 hours of reoxygenation (Hyp/Reox). The role of the MEK1/2-ERK1/2 cell survival pathway in ensuing 2'-MeCCPA mediated cardioprotection was assessed. The A_1AR agonist 2'-MeCCPA (10nM) was administered at 30 minutes post reoxygenation in the presence and absence of MEK 1/2 inhibitor UO126 (10µM). Results were shown as Mean±SEM and expressed as percentages of 10 000 total cells counted. *** $p<0.001$ vs. Normoxia. ### $p<0.001$ vs. Hyp/Reox. $$$ $p<0.001$ vs. 2'-MeCCPA at 30mins Post-R.

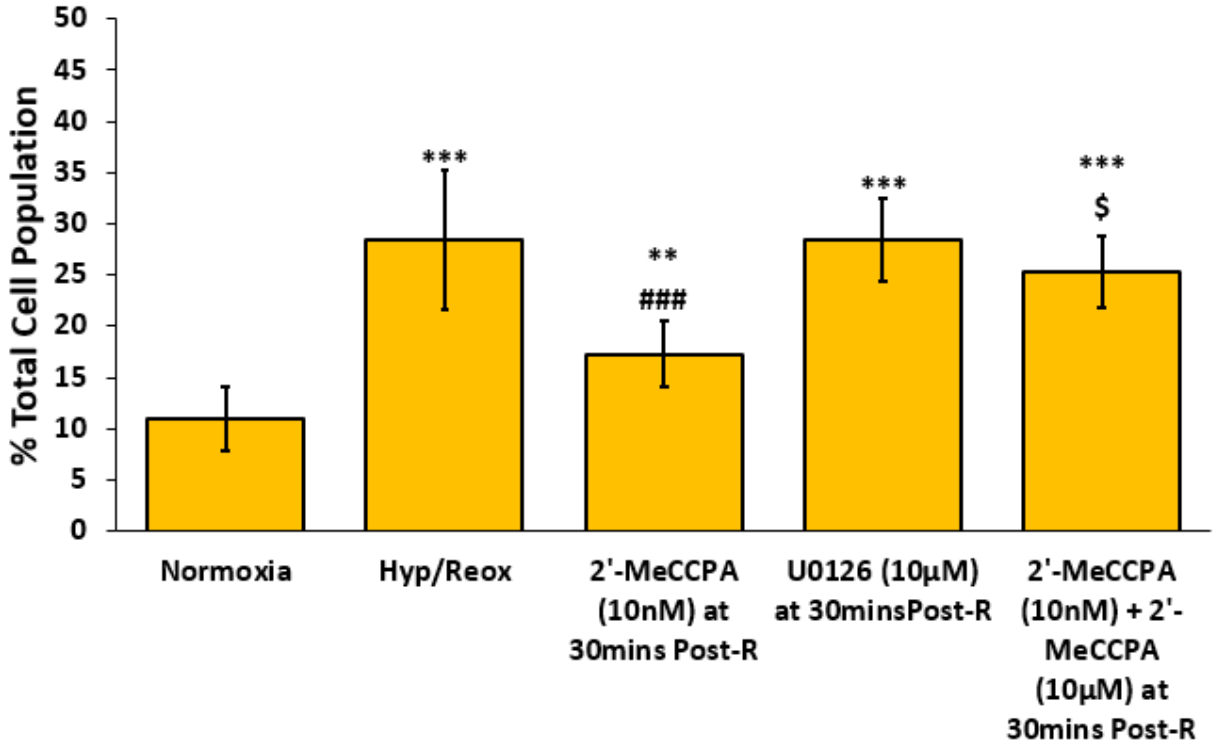


Figure 6. 18 The assessment of necrosis in isolated adult rat cardiac myocytes that were subjected to 4 hours of reoxygenation (Normoxia) or 1 hour of hypoxia and 3 hours of reoxygenation (Hyp/Reox). The role of the MEK1/2-ERK1/2 cell survival pathway in ensuing 2'-MeCCPA mediated cardioprotection was assessed. The A_1AR agonist 2'-MeCCPA (10nM) was administered at 30 minutes post reoxygenation in the presence and absence of MEK 1/2 inhibitor UO126 (10µM). Results were shown as Mean±SEM and expressed as percentages of 10 000 total cells counted. *** $p<0.001$ vs. Normoxia. ** $p<0.01$ vs. Normoxia. ### $p<0.001$ vs. Hyp/Reox. $ $p<0.05$ vs. 2'-MeCCPA at 30mins Post-R.

6.3.2.4a The effect of 2'-MeCCPA (10nM) when administration at 15 minutes post reoxygenation on cleaved caspase-3 activity in isolated adult rat cardiomyocytes via the recruitment of the MEK1/2-ERK1/2 cell survival pathway

It was previously found that when delaying the administration of 2'-MeCCPA (10nM) to 15 minutes post reoxygenation, there was a significant decrease in cleaved-caspase 3 activity compared to the Hyp/Reox control group (232±39% 2'-MeCCPA 15mins Post-R vs. 313±35% Hyp/Reox, $p<0.05$) (Figure 6.19).

In order to determine whether the decrease in cleaved-caspase 3 activity conferred by 2'-MeCCPA (10nM) being administered at 15 minutes post reoxygenation was via the

recruitment of the MEK1/2-ERK1/2 cell survival pathway; isolated rat cardiomyocytes were subjected to 1 hour of hypoxia and 3 hours of reoxygenation where A_1 adenosine receptor agonist 2'-MeCCPA (10nM) was administered at 15 minutes post reoxygenation in the presence and absence of MEK1/2 inhibitor UO126 (10µM).

The administration of 2'-MeCCPA (10nM) at 15 minutes post reoxygenation in the presence of MEK1/2 inhibitor UO126 (10µM) failed to significantly abolish the decrease in cleaved-caspase 3 that was observed when 2'-MeCCPA (10nM) was administered alone at 15 minutes post reoxygenation (293±32% 2'-MeCCPA+UO126 15mins Post-R vs. 232±39% 2'-MeCCPA 15mins Post-R, p>0.05) (Figure 6.19).

The administration of UO126 (10µM) alone at 15 minutes post reoxygenation had no significant effects upon cleaved-caspase 3 activity in comparison to the Hyp/Reox control group (312±35% UO126 15mins Post-R vs. 313±35% Hyp/Reox, p>0.05) (Figure 6.19).

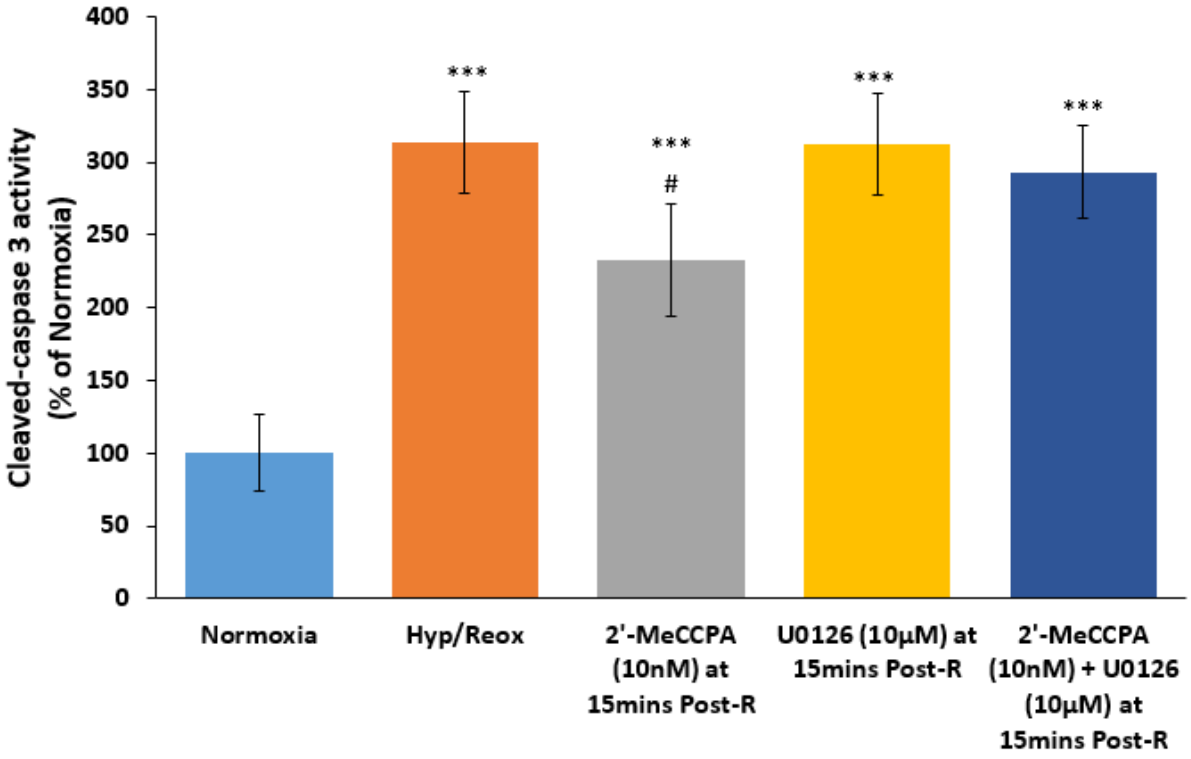


Figure 6. 19 Cleaved-caspase 3 activity within isolated adult rat cardiomyocytes subjected to 4 hours of oxygenation (Normoxia) or 1 hour of hypoxia followed by 3 hours of reoxygenation (Hyp/Reox). The A_1 agonist 2'-MeCCPA (10nM) was administered 15 minutes post reoxygenation in the presence and absence of the MEK1/2 inhibitor UO126 (10µM). Mean±SEM of 6 individual experiments. *** p<0.001 vs. Normoxia. # p<0.05 vs. Hyp/Reox.

6.3.2.4b The effect of 2'-MeCCPA (10nM) when administered at 30 minutes post reoxygenation on cleaved-caspase 3 activity in isolated adult rat cardiomyocytes via the recruitment of the MEK1/2-ERK1/2 cell survival pathway.

It was previously observed that when 2'-MeCCPA (10nM) was administered alone at 30 minutes post reoxygenation did not significantly reduce cleaved-caspase 3 activity when compared to the Hyp/Reox control group (263±43% 2'-MeCCPA 30mins Post-R vs. 313±35% Hyp/Reox, $p>0.05$) (Figure 6.20).

The administration of 2'-MeCCPA (10nM) at 30 minutes post reoxygenation in the presence of MEK1/2 inhibitor UO126 (10µM) failed to abolish the slight decrease observed when 2'-MeCCPA (10nM) was administered alone at 30 minutes post reoxygenation. There was no significant effect (300±16% 2'-MeCCPA+UO126 30mins Post-R vs. 263±43% 2'-MeCCPA 30mins Post-R, $p>0.05$) (Figure 6.20).

The administration of MEK1/2 inhibitor UO126 (10µM) alone at 30 minutes post reoxygenation had no significant effect upon cleaved-caspase 3 activity when compared to the control Hyp/Reox group (320±13% UO126 30mins Post-R vs. 313±35% Hyp/Reox, $p>0.05$) (Figure 6.20).

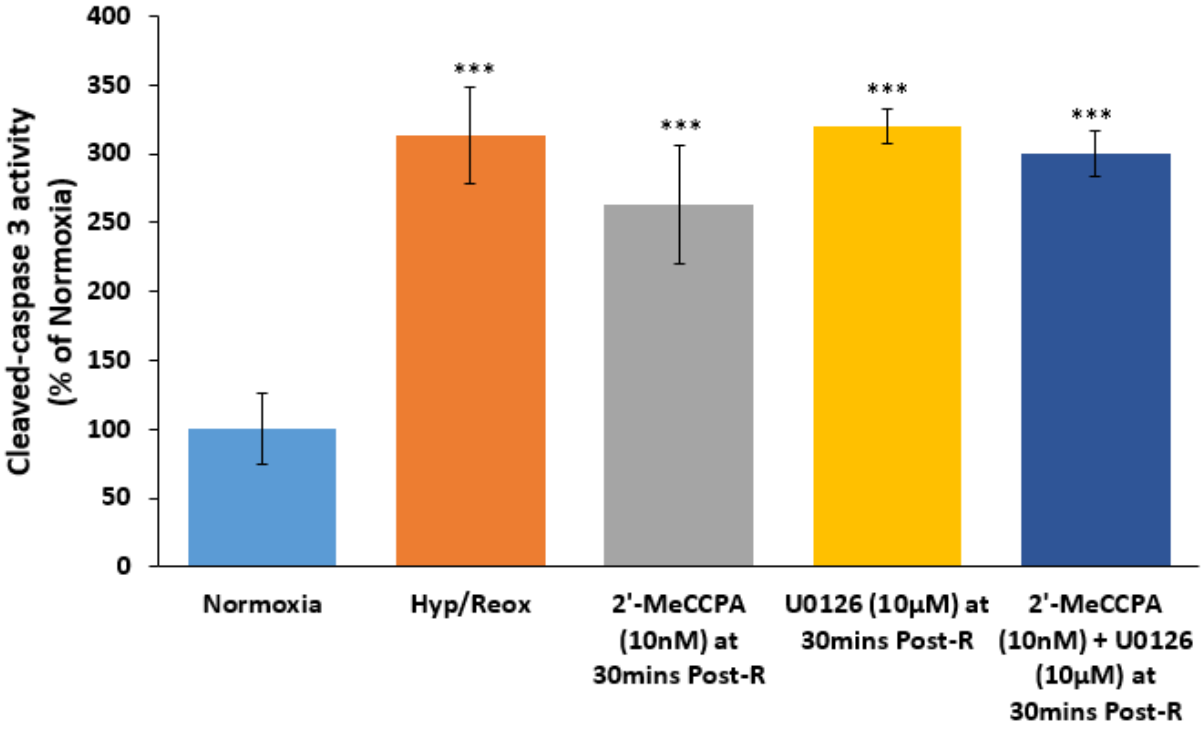


Figure 6. 20 Cleaved-caspase 3 activity within isolated adult rat cardiomyocytes subjected to 4 hours of oxygenation (Normoxia) or 1 hour of hypoxia followed by 3 hours of reoxygenation (Hyp/Reox). The A_1 agonist 2'-MeCCPA (10nM) was administered 30 minutes post reoxygenation in the presence and absence of the MEK1/2 inhibitor UO126 (10µM). Mean±SEM of 6 individual experiments. *** $p<0.001$ vs. Normoxia.

6.4 Discussion

The MEK1/2-ERK1/2 cell signalling pathway has been shown to play a vital role in cell survival, growth and differentiation. In context of cell survival, the phosphorylation of ERK1/2 can further activate downstream targets to further induce cardioprotection (Mocanu et al. 2002; Bose et al. 2005; Germack and Dickenson 2004). Our study is able to support the idea of cell survival through the upregulation of the MEK1/2-ERK1/2 cell survival pathway. When A_1 adenosine receptor agonist 2'-MeCCPA is administered at the onset of reperfusion/reoxygenation, it was able to significantly cause infarct limiting effects, decrease cell death (apoptosis and necrosis) and decrease cleaved caspase-3 activity. This therefore supports previous literature in explicitly showing that the MEK1/2-ERK1 cell signalling pathway can be activated by A_1 adenosine receptors and cause cardioprotection as seen in published literature by Germack and Dickenson (2004) who have shown that that stimulation of A_1 adenosine receptors using CPA can further activate ERK1/2 within new born rat cardiomyocytes which can then cause myocardium protection (Germack and Dickenson 2004). Our study can imply that the administration of A_1AR agonist 2'-MeCCPA at reperfusion/reoxygenation was able to decrease infarction and cell death via the recruitment of the MEK1/2-ERK1/2 cell signalling pathway; inhibition of MEK1/2 with UO126 abolished all cardioprotective effects. Our data therefore supports Germack and Dickenson (2004) in being able to upregulate the MEK1/2-ERK1/2 cell signalling pathway in order to confer cardioprotection.

Our study found that cleaved caspase-3 activity was seen to be reduced in response to A_1AR activation at the onset of reoxygenation in adult rat cardiomyocytes that were subjected to hypoxia/reoxygenation injury. This positive effect was abolished in the presence of MEK1/2 inhibitor UO126. This indicates that protection is dependent upon MEK1/2-ERK1/2 cell signalling pathway. Little research has been conducted into the A_1 adenosine receptor activation in order to ensure cardioprotection by the decrease in apoptosis and necrosis which can lead to a decrease in caspase-3 activity at the onset of reperfusion; however a similar methodology was conducted by Hussain et al. (2014) where the A_3 adenosine receptor was investigated. Similar results to our findings were observed when A_3AR agonist 2-CL-IB-MECA significantly reduced infarct size, upregulated p-ERK1/2 and reduced apoptosis,

necrosis and cleaved caspase-3 activity with manner that expressed the upregulation of the MEK1/2-ERK1/2 cell signalling pathway (Hussain et al. 2014). Although a different adenosine receptor subtype was investigated within the study, it drew similar conclusions to that of our study, implying that the activation of the A_1 adenosine receptor subtype can confer cardioprotection via the upregulation of the MEK1/2 cell signalling pathway. These similar findings could be due to the fact that the A_1 and A_3 adenosine receptor subtypes are vital receptors to ensure cardioprotection and when activated, they stimulate the MEK1/2 cell signalling pathway in similar ways as both receptors involve $G_{i/o}$ pertussis toxin proteins when activated (Germack and Dickenson 2004).

It was then important to look into if the MEK1/2 cell survival pathway mediated protection in a time dependent manner within the isolated rat model. Our study showed that post-reperfusion activation of the A_1ARs at 15 minutes and 30 minutes after the onset of reperfusion was seen to limit infarct size and decrease apoptosis and necrosis. These techniques were measured in accordance with MEK1/2 inhibitor UO126 can imply that when A_1 adenosine receptors were activated at 15 minutes and at 30 minutes post-reperfusion, a cardioprotective effect was observed. This is the first study to show that post-reperfusion activation of A_1AR at 15 minutes and 30 minutes post-reperfusion can protect the isolated rat heart and adult rat cardiomyocytes from ischaemic injury via the MEK1/2 cell signalling pathway. This therefore implies that in addition to the PI3K-AKT cell signalling pathway playing a vital role in cardioprotection, the MEK1/2 also indicates an alternative pathway to ensure cardioprotection.

From our study, post-reperfusion activation of the A_1 adenosine receptor allowed for infarct limiting effects and decrease in apoptosis and necrosis at the 15 minutes and 30 minutes time-point however a significant decrease in cleaved caspase-3 was not rendered significant during post-reperfusion activation of A_1ARs. This therefore implies that post-reperfusion cleaved caspase-3 activity could be independent of the MEK1/2 cell signalling pathway. This could be due to the fact that research by Hausenloy and Yellon (2004) has stated that most cardioprotection is conferred at the early stage of reperfusion and therefore 15 minutes or 30 minutes may be too late to administer A_1AR agonist 2'-MeCCPA to confer cardioprotection through the upregulation of the MEK1/2 cell signalling pathway to decrease cleaved caspase-3 activity.

Our findings within this study can be compared to those of Von Lubitz et al. (1994; 2001) as they looked into the administration of A_3AR 2-CL-IB-MECA administration at the onset of reperfusion or 20 minutes post-reperfusion in order to protect cerebral ischaemia reperfusion injury. Although we did not look into the A_3 adenosine receptor subtype, the delayed activation of the A_1 adenosine receptor at 15 minutes and 30 minutes post reperfusion was able to provide the same time dependent protection however in myocardial ischaemia reperfusion injury.

Hausenloy and Yellon (2004) stated that most protection occurs at early stages of reperfusion which is why administration of the A_1AR agonist 2'-MeCCPA was explored. The cardioprotective effects were observed to be high in a MEK1/2 cell signalling manner as infarct size was the most reduced. When there was a delayed administration of 2'-MeCCPA at 15 minutes and 30 minutes post-reperfusion, MEK1/2 cell signalling was still expressed however infarct size did increase compared to when 2'-MeCCPA was administered at reperfusion meaning that MEK1/2-ERK1/2 signalling could have decreased as timing then goes beyond the 'early phase of reperfusion'.

Further study that could have taken place was to look into the phosphorylation of p-ERK to detect if p-ERK activity was upregulated at the onset of reperfusion, 15 minutes and 30 minutes into the onset of reperfusion. This would have been useful and would have explored the ERK1/2 compartment to the MEK1/2-ERK1/2 cell signally pathway.

In light of current published literature and the data from this study, we can hypothesise and imply that cardioprotection can be associated with delayed activation of A_1ARs in the ischaemic reperfused myocardium and it may involve the upregulation of the pro-survival kinases.

6.5 Summary of Findings

To summarise this chapter, evidence can imply that the A_1 adenosine receptor agonist. 2'-MeCCPA has the ability to reduce myocardial ischaemia reperfusion injury in isolated perfused rat heart and adult rat cardiomyocyte models via the recruitment of the MEK1/2-ERK1/2 cell survival pathway through the activation of the A_1 adenosine receptor subtype at the onset of reperfusion but also through delayed activation at 15 minutes post-reperfusion and 30 minutes post-reperfusion. Our results show that:

- When A_1AR agonist 2'-MeCCPA was **administered at the onset of reperfusion**, this indicated that cardioprotection was conferred via the recruitment of the pro-survival MEK1/2-ERK1/2 cell signalling pathway. This was observed through the limiting infarct size effects, a decrease in apoptosis and necrosis, and a decrease in cleaved caspase-3 activity which was all reversed in the presence of MEK1/2 inhibitor UO126.
- When delayed activation of A_1 adenosine receptors by the administration of A_1AR agonist 2'-MeCCPA at **15 minutes post-reperfusion** occurred, this conferred cardioprotection via the recruitment of the MEK1/2-ERK1/2 pro-survival pathway. This was observed through the limitation in infarct size, decreases in apoptosis and necrosis. An upregulation in p-ERK phosphorylation was also observed; all of which was reversed in the presence of MEK1/2 inhibitor UO126. There was no significant effect observed on cleaved caspase-3 activity.
- Further delayed activation of A_1 adenosine receptors occurred where A_1AR agonist 2'-MeCCPA was administration **30 minutes post-reperfusion** which showed cardioprotective effects via the recruitment of the MEK1/2-ERK1/2 cell signalling pathway. A limiting effect in infarct size was observed as well as a decrease in apoptosis and necrosis. These effects were then reversed in the presence of MEK1/2 inhibitor UO126. No effect was observed on cleaved caspase-3 activity.

Chapter 7: General Discussion

7.1 Discussion

Coronary heart disease is one of the leading causes of death within the UK and has the ability to affect individuals' quality of life. With an increased risk of mortality due to coronary heart disease (CHD), an increase in research to reduce the prevalence of CHD is required (Department of Health 2018). In order to reduce the burden of coronary heart disease, policies have been put in place that identify high risk patients and treat them as early as possible (Skinner et al. 2007). Examples of these policies are patients over the age of 40 are required to attend regular checks by their GP (Skinner et al. 2007). The needs for novel therapies in order to reduce the damaging effects of a myocardial infarctions are still extensively being researched.

Furthermore within a clinical setting, patients are often admitted following the development of angina or after having suffered from a myocardial infarction. Cardiac troponin and creatinine kinase levels are assessed to further determine the diagnosis of a myocardial infarction, an ECG is also conducted to confirm a diagnosis (Collinson and Gaze 2007). Following the diagnosis of myocardial infarction patients may undergo thrombolytic therapy, primary angioplasty or even coronary bypass grafting in order to restore blood flow back to the ischaemic region (Collinson and Gaze 2007).

The restoration of coronary blood flow to the ischaemic myocardium is a phenomenon called reperfusion (Hausenloy and Yellon 2004). It remains to be one of the mechanisms of salvaging any reversible damage to cardiomyocytes after the injurious ischaemic event however reperfusion itself can also further the injury process too (Hausenloy and Yellon 2004); therefore due to this reason, it is essential to elucidate the mechanisms that contributes to the hastening of the injury process within this period but also imperative to research the potential targets within the reperfusion period in order to develop and research cardioprotective agents that could potentially limit ischaemia reperfusion injury when administered in a clinical setting. Many advances over time have led to better understanding in the signalling process to mediate myocardial ischaemia reperfusion injury (Hausenloy and Yellon 2004). A multitude of studies have also researched how endogenous and exogenous

agents can mediate ischaemia reperfusion injury, having detrimental effects; others can possess innate cardioprotective abilities (Hausenloy and Yellon 2004).

When the myocardium becomes ischaemic there is insufficient availability of oxygen and nutrients in order to maintain oxidative phosphorylation and therefore intracellular adenosine levels increase following the breakdown of ATP and APD (Headrick et al. 2011). Adenosine has been described as a 'retaliatory metabolite' as it is a nucleoside that is released when in response to metabolically compromised cells (Headrick et al. 2011). Adenosine is known to exert its physiological effects via four know adenosine receptors which include; A_1, A_{2A}, A_{2B} and A_3 receptor subtypes. The A_1 and A_3 receptor subtypes are coupled with $G_{i/o}$ proteins whereas the A_{2A} and A_{2B} receptor subtypes are coupled to the $G_{s/olf}$ proteins (Sheth et al. 2014) which therefore could imply that the A_1 and A_3 receptors could function in similar ways.

The A_1 receptor subtype was the first adenosine receptor to be implicated in the cardioprotective effects of adenosine (McIntosh and Lasley 2011). The initial evidence of expression of A_1 adenosine receptors was initiated by Lohse et al. (1987) where the investigation of the expression of A_1 adenosine receptors in mammalian ventricular myocardium was conducted. This was then later investigated in 1988 and further confirmed that A_1 adenosine receptors are expressed within isolated rat ventricular cardiomyocytes (Martens et al. 1988). The A_1 adenosine receptor subtype was also known to be expressed on coronary vascular smooth muscle and cardiac fibroblasts in animal models (McIntosh and Lasley 2011). With this background information of the A_1 adenosine receptor subtype being present in isolated rat myocardium, it became an area of interest within this study to look specifically at the effects of the A_1 adenosine receptor activation at various time points post reperfusion and to determine their associated cell signalling pathways.

Furthermore, research conducted in 1990 provided the first form of evidence for the stimulation of A_1 adenosine receptors with the use of an A_1 adenosine agonist named R-N6-(phenyl-2R-isopropyl)-adenosine (PIA) whilst investigating the mechanism associated with pharmacological preconditioning. PIA was administered prior to ischaemia in isolated rat hearts and was associated with infarct sparing effects (Lasley et al. 1990). The study concluded the activation of A_1 adenosine receptors having the ability to confer

cardioprotection. With this earlier information provided, the interest into A_1 adenosine receptor agonists in causing cardioprotection became another area of interest.

The findings from the current study further imply that the activation of the A_1 adenosine receptors at the onset of reperfusion or delaying the activation to 15 or 30 minutes post-reperfusion can also ameliorate the detrimental effects of ischaemia reperfusion. To further support that the A_1 adenosine receptor played a major part in the protection observed, the A_1AR antagonist DPCPX was co-administered with A_1 adenosine receptor agonist 2'-MeCCPA at either reperfusion, 15 or 30 minutes post-reperfusion, DPCPX reversed all cardioprotection. The findings from this current study support the activation of A_1 adenosine receptors in ameliorating myocardial ischaemia reperfusion injury.

Our study implies that when the A_1AR agonist 2'-MeCCPA was administered at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion, sufficient cardioprotection was observed via the significant decrease in infarct size, a reduction in apoptosis and necrosis as well as decreased cleaved caspase-3 activity and an upregulation in p-AKT phosphorylation (visually represented in Figure 7.1 below). These cardioprotective effects were reversed in the presence of PI3K inhibitor Wortmannin, indicating that 2'-MeCCPA mediated cardioprotection involved the recruitment of the PI3K-AKT pro-survival pathway. This cell survival signalling pathway has the ability to decrease pro-apoptotic proteins such as caspases and increase pro survival molecules such as AKT, endothelial nitric oxide synthase (eNOS) as well as p70S6K (Hausenloy and Yellon; Mocanu and Yellon 2004). Previous studies have not explicitly shown the administration of A_1 adenosine receptors at the onset of reperfusion within the heart model however this has been investigated within the lung ischaemia-reperfusion injury model and shown to cause protective effects (Gazoni et al. 2010). Previous studies have shown A_1AR agonist to mediate cardioprotection via the PI3K-AKT signalling pathway however this is the first study to show cardioprotection conferred by an A_1AR agonist being administered at the onset of reperfusion and post-reperfusion. Wortmannin, a well characterised PI3K inhibitor has previously shown to ameliorate cardioprotection in an ischaemia reperfusion setting and therefore used within this current study to evaluate the involvement of the PI3K-AKT signalling pathway (Hussain et al. 2014).

Our study further observed the role of the MEK1/2 pro-survival pathway in A_1 adenosine receptor mediated cardioprotection. As previously mentioned, when A_1AR agonist 2'-

MeCCPA (10nM) was administered at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion; this delayed activation of the A_1 adenosine receptor subtype mediated cardioprotection via a limit in infarct size, reducing apoptosis and necrosis leading to a reduction in cleaved caspase-3 activity. These protective effects were reversed in the presence of MEK1/2 inhibitor UO126. Although, the decrease in cleaved caspase-3 activity seen when 2'-MeCCPA was administered at 15 or 30 minutes post reperfusion was not reversed by UO126 indicating that the anti-caspase 3 activity effects were more specifically associated with the PI3K cell signalling pathway. Germack and Dickenson (2004) have shown that ERK1/2 phosphorylation was upregulated with the activation of the A_1 adenosine receptors via CPA administration in new born rat cardiomyocytes via the pre-conditioning phenomenon (Germack and Dickenson 2004) which could support that the MEK1/2 cell survival pathway may be upregulated throughout pre-conditioning but maybe not throughout post-reperfusion activation of A_1ARs.

These findings support the research conducted by Hausenloy and Yellon (2004) who stated that targeting anti-apoptotic mechanisms at the time of reperfusion and the early stages of reperfusion, was a potential approach in attenuating reperfusion-induced cell death. They further associate the ERK1/2 signalling pathway with cardioprotection.

The A_1 and A_3 adenosine receptors activate in such a similar way that when activated they cause a decrease in cyclic AMP activation whereas A_{2A} and A_{2B} increase cyclic AMP production. Therefore similarities could be drawn upon between the A_1 and A_3 receptor subtypes in terms of the fact that the A_3 adenosine receptor has a more extensive researched background rather than the A_1 receptor subtype. A study conducted by Hussain et al (2014) investigated the effects of A_3AR mediated cardioprotection. It was found that the A_3ARs, when activated in adult rat cardiomyocytes, decreased caspase-3 activity however this implicated the upregulation of the PI3K-AKT and MEK1/2-ERK1/2 cell signalling pathways. Drawing these conclusions from the research conducted by Hussain and colleagues (2014), similar findings were detected within this current study when the A_1 adenosine receptor was investigated implying that the upregulation of the PI3K-AKT and MEK1/2-ERK1/2 cell signalling pathways suggested cardioprotective effects in an A_1AR activation manner.

The PI3K-AKT and MEK1/2-ERK1/2 pro-survival pathways make up two branches of the Reperfusion Injury Salvage Kinase (RISK) pathway and pervious research has suggested that

both branches can be activated to protect following a lethal ischaemic insult (Hausenloy and Yellon 2004). Previous studies have shown how insulin , insulin-like growth factors (IGF-1), transforming growth factor-β1 (TGF- β1), urocortin, atorvastatin and bradykinin to protect the heart by the upregulation of both/or the PI3K-AKT and MEK1/2-ERK1/2 pathways (Hausenloy and Yellon 2004). Our findings therefore support that the A_1AR agonist 2'-MeCCPA, when administered at reperfusion causes both the PI3K and MEK1/2 pathways to upregulate to enhance protection after the ischaemic insult that has taken place.

Our group has previously shown A_3AR agonist 2-CI-IBMECA to limit reperfusion injury throughout anti-apoptotic and anti-necrotic mechanisms (Hussain et al. 2006). It was also shown that when activating A_3 receptors at different time points throughout reperfusion (15 or 30 minutes), this resulted in reduced myocardial injury and was dependent on AKT and ERK1/2 activation. Rat hearts were subjected to 35 minutes of coronary artery occlusion followed by 120 minutes of reperfusion where 2-CI-IBMECA was administered at reperfusion, 15 or 30 minutes post reperfusion. This protocol is similar to the current project due to when the A_1AR agonist 2'-MeCCPA was administered at reperfusion, 15 minutes or 30 minutes post reperfusion, there was a significant decrease upon infarct size and this was then reversed in the presence of UO126 (MEK1/2 inhibitor) which further suggests that the MEK1/2 signalling pathway does play a part to the cardioprotection observed. The PI3K-AKT signalling pathway was also depended on in a time point manner to ensure cardioprotection.

A study by Lubitz et al (2001) also investigated that when A_3AR agonist IB-MECA was administrated 20 minutes into the onset of reperfusion, a significant decrease in infarct volume within the brain was detected which also proved that the concept of post-reperfusion activation of adenosine receptors confers cytoprotective effects.

7.2 Evaluation of pharmacological adenosine preconditioning and post conditioning in comparison to delayed reperfusion A_1 adenosine receptor activation

Endogenous and exogenous agents, especially adenosine, has been investigated within mechanical mechanisms; such as the phenomenon of pre-conditioning and post-conditioning to ensure that ischaemia reperfusion injury is salvaged. Pre-conditioning is an intrinsic process in which repeated short episodes of ischaemia are induced to prevent the long term damage

caused by prolonged episodes of ischaemia (Iliodromitis et al. 2007; Singh et al. 2018). With ischaemic preconditioning being such a powerful tool, researchers have demonstrated that adenosine plays an important role within protection of the myocardium in remote preconditioning (McCully et al. 2001; Singh et al. 2018). Therefore in order to elucidate the mechanisms that were involved in adenosine mediated cardioprotection in a preconditioning setting, scientists further administered adenosine exogenously which is known as adenosine pharmacological preconditioning (Singh et al. 2018). It was furthermore shown that preconditioning studies involving adenosine induced cardioprotection by various mechanisms that included the activation of the K_{ATP} channels in rat and rabbit species (Baxter and Yellon 1999; Takano et l. 2001; Singh et al. 2018). Interaction with various kinases were also investigated for example PKC, MAP Kinase, MEK 1, ERK1/2 and tyrosine kinases within rat and rabbit species (Kudo et al. 2002; Dana et al. 2000; Germack and Dickenson 2005; Zhao et al. 2001; Ballard-Croft et al. 2005; Williams-Pritchard et al. 2011). With the phenomenon of pharmacological preconditioning with adenosine being able to activate various mechanisms to promote myocardial salvage, it was important to investigate the various mechanisms that become activated throughout delayed reperfusion A_1 adenosine receptor activation to explore if similar pathways were able to ensure cardioprotection. For this reason, the PI3K and MEK1/2 signalling pathways were investigated within this current study.

With adenosine having the ability to induce cardioprotection, the A_1 adenosine receptor is an important receptor and area of research within this project and previous research by Germack and Dickenson (2005) showed that A_1 mediated adenosine receptor activation through the phenomenon of preconditioning ensured cardioprotection in neonatal rat cardiomyocytes. The A_1 agonist of choice within this study was CPA. Therefore with proven research showing the effects of the A_1 receptor ensuring cardioprotection through pharmacological preconditioning, it was interesting to investigate the effects of the delayed reperfusion activation of the A_1 adenosine receptor to ensure cardioprotection through the PI3K and MEK1/2 signalling pathways.

Preconditioning with adenosine comes with its own share of disadvantages and an important one is that the clinical application is limited due to ethical and practical reasons (Andreadou et al. 2008). Although this phenomenon has an ability to limit infarct size, we can't apply it to patients as we do not know if they are or are not going to encounter a cardiac event

(Andreadou et al. 2008). Therefore our current project is useful in bringing forward research that involves post-reperfusion activation of A_1 adenosine receptors in order to salvage the myocardium from ischaemia reperfusion injury. The clinical relevance of this study suggests that if patients have already endured a myocardial infarction then administering a therapeutic agent that targets the A_1 adenosine receptors and ensuring cardioprotection up to 30 minutes after a patient has had an ischaemic insult, could prove beneficial and an area for further research beyond the scope of this current project.

With preconditioning being such an extensively researched phenomenon within an experimental setting, post conditioning is also a very interesting concept that has been extensively researched. This is when brief periods of coronary occlusions are performed just at the beginning of reperfusion (Pagliaro and Penna. 2011). Adenosine has been said to play a part in the post conditioning phenomenon however a study by Kin et al (2005) have shown that the A_2 adenosine receptor plays an important role in the protective effects within mouse hearts rather than the A_1 adenosine receptor (Kin et al. 2005). Previous researchers have shown that pharmacological post conditioning with adenosine can activate the RISK (PI3K-AKT and MEK1/2-ERK1/2 signalling pathways) and SAFE pathways in order to ensure cardioprotection (Shi and Vinten-Johansen 2012; Procopio et al. 2020). So therefore it could mean that endogenous adenosine can ensure cardioprotection but through the A_2 adenosine receptor subtype (Kin et al. 2005). However researchers such as Xi et al (2008) researched that distinctive strains of gene knockout mice, post conditioning induced infarct-limiting cardioprotection was conferred and this was triggered by the activation of the A_1 adenosine receptor (Xi et al. 2008). This suggests that there is confounding research when it comes to post conditioning and the activation of the A_1 adenosine receptor. So having this current project highlighting the importance of post-reperfusion activation of the A_1ARs is another important phenomenon which can ensure cardioprotection.

7.3 Haemodynamic Studies (Coronary Flow, Left Ventricular Developed Pressure and Heart Rate)

Within this study, minimal significance was shown within haemodynamic data which included coronary flow, left ventricular developed pressure and heart rate when 2'-MeCCPA (10nM) was administered. This could have been due to the fact that such a low concentration of the A_1 adenosine agonist 2'-MeCCPA was used. It was shown that the concentration of 10nM made a significant effect upon infarct size, cell death and caspase-3 activity however there was little/no effect upon haemodynamic data. This could mean that for haemodynamic parameters to efficiently be investigated, higher concentrations of the A_1 adenosine receptor agonist 2'-MeCCPA could have been investigated in order to detect significance in parameters. This could support the fact that in Chapter 2, the higher concentration of 100nM of 2'-MeCCPA caused a decrease in HR only.

Using the Langendorff technique itself can be unreliable and it comes with its own set of disadvantages. The longer the hanging process of the heart takes, the more damage the rat hearts could have incurred and therefore can lead to further ischaemic damage to the heart. Additionally, the exposure of the heart to our natural environment without any sort of protective element to it could contribute to tissue temperature changes and ischaemic damage which could therefore cause inconsistencies within results (Motayagheni 2017). Therefore due to the fact that the Langendorff may not be the most reliable technique to measure haemodynamic parameters, further damage may have been caused and this could have led to the insignificant results detected within the haemodynamic parameter results within this project.

Within this current study, Figure 3.2. has shown that when the concentration of 100nM of 2'-MeCCPA was administered at the onset of reperfusion, there was a consistent decrease in heart rate, which was not detected when the 10nM concentration of 2'-MeCCPA was administered. This effect of the slowing of heart rate can be compared to a study conducted by Koeppen et al. in 2009 where they showed that ex vivo studies implicated the A_1 adenosine receptor in the slowing down of the heart rate (Koeppen et al. 2009). More importantly, studies conducted by Albrecht-Küpper et al. in 2011 found that when administering A_1 adenosine agonist CCPA to male Wistar rats at increasing concentrations of 1nm – 1μM, the

higher concentrations resulted in a reduced heart rate within isolated rat Langendorff hearts. Higher concentrations even caused complete AV block (Albrecht-Küpper et al. 2011). This could therefore support why at the concentration of 100nM of 2'-MeCCPA, there was a reduced heart rate within the reperfusion period when 2'-MeCCPA (100nM) had been administered. With this being said, presumably the effects of 2'-MeCCPA at the concentrations of 1µM had also been explored and no significant difference in heart rate was detected when compared to the IR control; which leads to believe that potentially 2'-MeCCPA may not have been a fully selective A_1 adenosine agonist when compared to A_1AR agonist such as CCPA (Albrecht-Küpper et al. 2011). This also leads to believe that more experimental research could have been conducted to look into why the heart rate didn't cause a significant change at the highest concentration of 2'-MeCCPA (1µM).

Furthermore, this current study showed no significant effect upon heart rate was detected when A_1 adenosine antagonist DPCPX was administered alongside A_1AR agonist 2'-MeCCPA throughout the project and this can be supported by published literature by Koeppen et al. in 2009 which showed that when DPCPX was administered to A_1ARs, there was no effect upon heart rate (Koeppen et al. 2009).

This current project showed that there was no significant differences in left ventricular developed pressure (LVDP) and coronary flow (CF) when A_1AR agonist was administered to the isolate perfused rat hearts throughout the period of reperfusion. After ischaemia was induced a general decrease in LVDP and CF was observed and this can be supported by a study by Lozza et al. (1997) where they showed that when perfused hearts underwent ischaemia, there was a general decrease in LVDP and CF by around 50-75% compared to the control group. Lozza et al. (1997) also went on to find that when reperfusion commenced, the perfused hearts administered with CCPA (3nM) allowed for immediate LVDP recovery immediately after coronary flow was restored. This supports our findings as it shows that A_1 adenosine agonists have the ability to restore LVDP and CR after an ischaemic event and therefore beneficial towards perfused rat hearts.

7.4 Study Limitations and Further Investigations

Although cross talk between the two branches of the RISK pathway was not something that was investigated within this study; previous research has touched on the fact that there is cross-talk interaction between the PI3K-AKT and MEK1/2-ERK1/2 pro-survival pathways. A study conducted by Hausenloy et al. (2004) showed that cross-talk between the two branches of the RISK pathway did occur at the early reperfusion in an ischaemic preconditioning setting within isolated perfused rat hearts. Therefore it would be useful to investigate if cross-talk occurred within our study in the phenomenon of delayed reperfusion activation of A_1 adenosine receptors. When UO126 (10µM) and 2'-MeCCPA (10nM) was administered together at 15 minutes and 30 minutes post-reoxygenation, it was unable to block the protective effects shown on cleaved-caspase-3 activity suggesting that there was little effect seen from the MEK1/2-ERK1/2 cell signalling pathway. This could also suggest that potentially cross-talk was occurring as similar experiments to explore the PI3K-AKT cell signalling pathway at the 15 minutes and 30 minute time-points were carried out in this study and the PI3K-AKT sell signalling branch of the RISK pathway was upregulated at these time-points in a caspase-3 manner whereas the MEK1/2-ERK1/2 cell signalling pathway was not. Further investigation into cross-talk between the two branches of the RISK pathway could be explored.

With ample research being conducted into the RISK pathway, our findings imply that A_1 adenosine activation recruits the PI3K-AKT pathway in a delayed reperfusion activation manner however the MEK1/2-ERK1/2 pathway is also implicated alongside. Further investigations can be conducted beyond the RISK pathway to explore the activation of the novel protective pathway named Survivor Activating Factor Enhancement (SAFE) pathway (Lecour 2009).

Other cell survival pathways have been involved in mediating cell survival and further studies are required in order to determine if they have a part to play towards cardioprotection. These cell signalling cascades can include; p38 and PKC. The survivor activating factor enhancement (SAFE) pathway is an alternative intrinsic pro-survival signalling pathway to the RISK pathway for cardioprotection. Therefore further studies can be conducted in order to investigate the above cell signalling pathways with the activation of A_1 agonist 2'-MeCCPA mediating

cardioprotection. Another A_1 agonist, CCPA can also be investigated with the above cell signalling pathways to determine if cardioprotection is enhanced. Recent has shown that the Wnt/β-catenin pathway could be considered as a therapeutic target when activated by adenosine however more research needs to be conducted (Procopio et al. 2020).

When the assessment of various concentrations of A_1 adenosine receptor (A_1AR) agonist, 2'-MeCCPA was conducted, the concentrations of 100nM and 1μM also displayed effective cardioprotective effects by reducing infarct size to risk ratio (%) within an isolated rat's heart. It would have been useful to further examine the effects of this higher concentrations (100nM and 1μM) rather than 10nM to investigate their effects upon the PI3K-AKT and MEK1/2-ERK1/2 cell signalling pathways.

When the activation of A_1ARs was delayed with 2'-MeCCPA (10nM) to 60 minutes post-reperfusion, there was a failure in protecting the Langendorff rat heart from ischaemia reperfusion injury. Further studies could be carried out in order to determine if higher concentrations of 2'-MeCCPA administered at 60 minutes post-reperfusion make a difference in protecting the ischaemic-reperfused myocardium from injury.

There are a variety of different potent A_1 adenosine agonists that have been reported in literature, therefore it was would useful in determining the different effects of the different A_1 agonists to ensure that they all contribute towards cardioprotection and if there are other agonists that ensue more cardioprotective that 2'-MeCCPA.

There are previous studies that report A_3AR agonist 2-CL-IB-MECA to activate AKT and ERK1/2 in a concentration/time dependent manner and also to be pro- and anti-apoptotic (Mocanu et al. 2002). In this current study, it has been established that the A_1AR agonist 2'-MeCCPA can activate PI3K and MEK1/2 in a time and concentration dependent manner but also further studies may be required to determine the threshold that is required for AKT and MAPK1/2 activation by 2'-MeCCPA to observe cardioprotection instead of just using one concentration of 10nM.

Phosphorylation of p-ERK1/2 could have been fully explored within this study to detect if there was an upregulation at the onset of reperfusion as well as 15 and 30 minutes post-reperfusion.

In order to determine the activity of caspase 3 in this current study, the activity of active/cleaved caspase 3 was assessed. The activation of caspase-3 requires the proteolytic processing of the inactive zymogen into cleaved/activated p16 and p12 fragments. In our study we used the cleaved-caspase 3 antibody to detect the endogenous levels of cleaved-caspase 3. This antibody is not able to fully recognise the full length of caspase 3 or other caspases therefore the data needs to be interpreted with caution as it is not a direct measure upon caspase 3 itself. Further study needs to be conducted.

This overall project hightlights the importance of the delayed activation of A_1ARs within the reperfusion period in a rat model and how this can could be taken further for future research and extrapolating into a clinical setting. The specific signalling pathways, for example the PI3K and MAPK1/2 cell signalling pathways were implicated within A_1AR activation cardioprotection and further research and study would need to be conducted to fully evaluate the extent of the usefulness of these cell signalling pathways when it comes to ensuring cardioprotection.

7.5 Conclusion

The work carried out in this thesis has successfully implied the cardioprotective effects of the activation of A_1 adenosine receptors in an ischaemia/reperfusion injury model. This is the first study to imply the delayed activation of A_1 adenosine receptors with A_1AR agonist 2'-MeCCPA to confer cardioprotection in isolated perfused rat hearts. This cardioprotective effect is associated with decreased infarct size (%) and decreased apoptosis and necrosis via the recruitment of the PI3K and MEK1/2 cell survival pathways, this has been visually represented in the graphical abstract, Figure 7.1 below. The findings from this study indicate clinically important developments in the field of managing myocardial infarction. Urgent studies are required to investigate the potential role of A_1 adenosine receptor agonists in ameliorating myocardial ischaemia reperfusion injury in human.

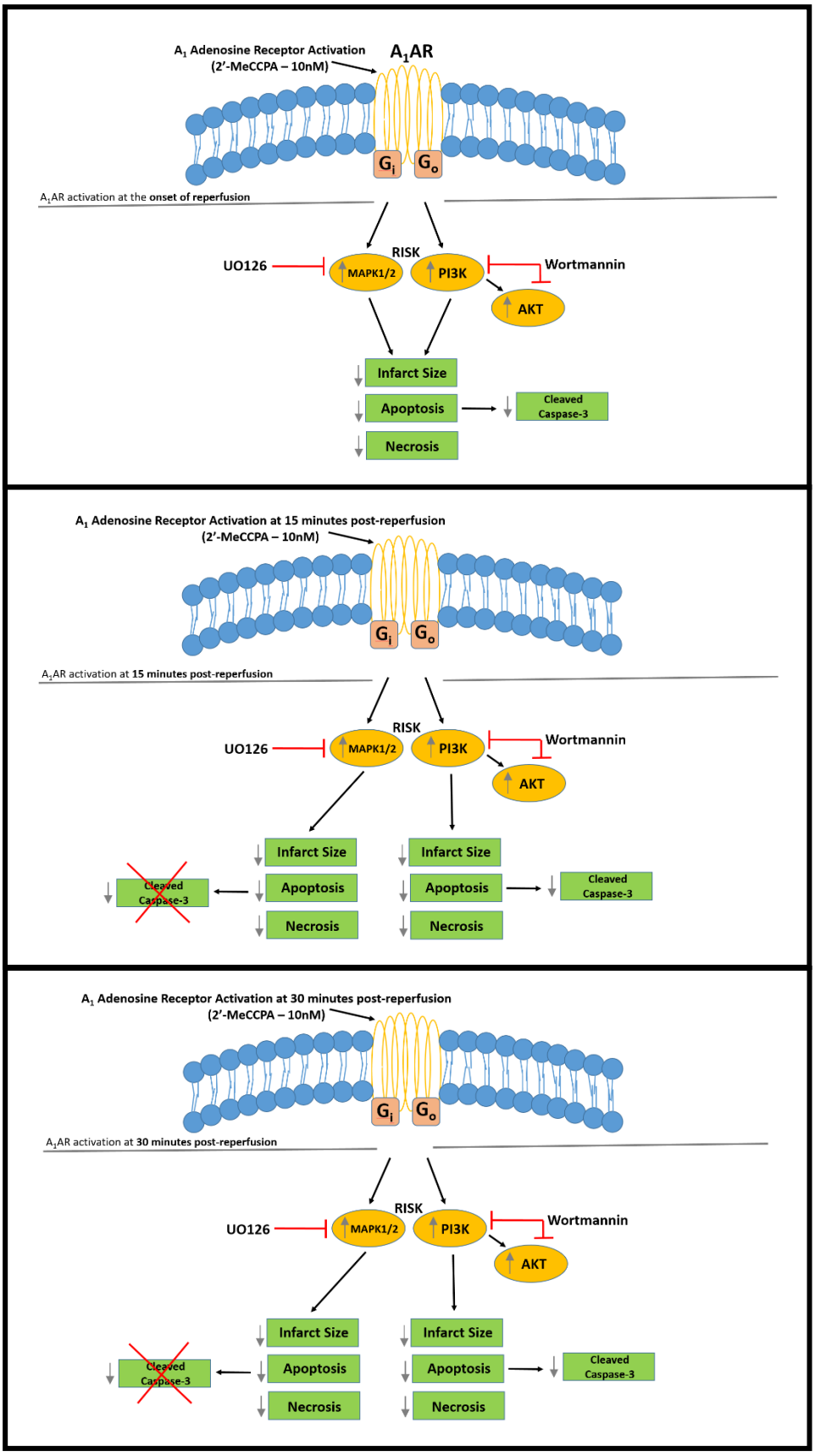

A1 Adenosine Receptor Activation
(2'-MeCCPA – 10nM)
A1AR
Gi
Go
A1AR activation at the onset of reperfusion
RISK
UO126
MAPK1/2
PI3K
Wortmannin
AKT
Infarct Size
Apoptosis
Cleaved Caspase-3
Necrosis
A1 Adenosine Receptor Activation at 15 minutes post-reperfusion
(2'-MeCCPA – 10nM)
A1AR activation at 15 minutes post-reperfusion
A1 Adenosine Receptor Activation at 30 minutes post-reperfusion
(2'-MeCCPA – 10nM)
A1AR activation at 30 minutes post-reperfusion

Figure 7. 1 Graphical abstract to summarise the key findings and the cell signalling pathways that were implicated in ensuring cardioprotection. When A_1AR agonist 2'-MeCCPA (10nM) was administered at the onset of reperfusion, it can be implied that cardioprotection has occurred through the activation of the PI3K and MAPK1/2 cell signalling pathways. This was represented by a decrease in infarct size (%) as well as a decrease in apoptosis and necrosis leading to a decrease in cleaved caspase-3 activity. When 2'-MeCCPA (10nM) was administered at 15 minutes and 30 minutes post-reperfusion, the same effects can be implied however a decrease in cleaved caspase-3 was not detected.

The first results chapter (Chapter 3) successfully implied that the concentration of 10nM of A_1AR agonist 2'-MeCCPA, when administered at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion, protected an isolated perfused rat heart undergoing simulated ischaemia/reperfusion as well as in rat cardiomyocytes that underwent hypoxia/reoxygenation. There was a decrease in infarct size, a decrease in cell death and cleaved caspase-3 activity.

Chapter 4 determines that the A_1 receptor in particular plays a vital role in the conferred cardioprotection that is shown in this study. A_1AR agonist is administered alongside A_1AR antagonist DPCPX and this antagonist blocks all protective effects seen when the A_1 adenosine receptor is activated implying that the A_1 adenosine receptor is vital for cardioprotection. This is apparent at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion. DPCPX abrogates the limit in infarct size, decrease in cell death and cleaved caspase-3 activity, which has been visually represented in Figure 7.1. Chapter 4 also investigates the upregulation of AKT phosphorylation which in turn implies the PI3K-AKT cell signalling pathway is playing a vital role to ensure cardioprotection via the A_1 adenosine receptor.

Furthermore, Chapter 5 investigates how the PI3K cell survival pathway is upregulated by the activation of the A_1AR by 2'-MeCCPA and the protection conferred in reversed with the PI3K inhibitor Wortmannin. This shows the direct link between the upregulation of the PI3K cell survival pathway and the activation of the A_1 adenosine receptors at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion/reoxygenation as seen in Figure 7.1.

Lastly, Chapter 6 investigated the role of the MEK1/2 cell survival pathway when the A_1ARs are activated. It was found that when 2'-MeCCPA was administered at the onset of reperfusion, 15 minutes and 30 minutes post-reperfusion/reoxygenation; there was a decrease in infarct size and cell death and this was abrogated in the presence of MEK1/2

inhibitor UO126. However this reverse was not observed when UO126 was administered alongside 2'-MeCCPA on cleaved caspase-3 activity at 15 minutes and 30 minutes post-reoxygenation. Therefore this could imply that the PI3K branch of the RISK pathway plays more of a role in cardioprotection conferred by the activation of the A_1 adenosine receptors rather than the MEK1/2 branch of the RISK pathway.

Chapter 8: References

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